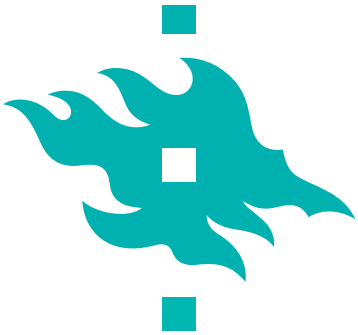

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

L. LAURIINA POROKUOKKA

THE ROLE OF GDNF AND ITS RECEPTOR GFRa1 IN NEURONAL DEVELOPMENT AND FUNCTION

INSTITUTE OF BIOTECHNOLOGY
HELSINKI INSTITUTE OF LIFE SCIENCES AND
DIVISION OF PHARMACOLOGY AND PHARMACOTHERAPY
FACULTY OF PHARMACY
DOCTORAL PROGRAMME IN BIOMEDICINE
UNIVERSITY OF HELSINKI

The role of GDNF and its receptor GFRa1 in neuronal development and function

L. Lauriina Porokuokka

Department of Pharmacology,
Faculty of Medicine
and
Helsinki Institute of Life Sciences
and
Institute of Biotechnology
and
Division of Pharmacology and Pharmacotherapy,
Faculty of Pharmacy

Graduate Program in Biotechnology and Molecular Biology,
Doctoral Program in Biomedicine

University of Helsinki
Finland

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Pharmacy,
University of Helsinki, for public examination in Auditorium 107, Athena, Siltavuorenpenger 3 A,
Helsinki, on May 20th, 2020, at noon.

Supervisors Associate Professor Jaan-Olle Andressoo, PhD
Department of Pharmacology
Faculty of Medicine, HiLIFE
University of Helsinki

Professor Raimo K. Tuominen, MD, PhD
Division of Pharmacology and Pharmacotherapy
Faculty of Pharmacy
University of Helsinki

Reviewers Assistant professor Meenakshi Rao, MD, PhD
Division of Gastroenterology, Hepatology and Nutrition
Boston Children's Hospital
Department of Pediatrics, Harward Medical School
Boston, MA, USA

Docent Annika Meinander, PhD
Department of Cell Biology
Faculty of Science and Engineering, BioCity
Åbo Akademi University

Opponent PhD Ulrika Marklund
Division of Molecular Neurobiology
Department of Medical Biochemistry and Biophysics
Karolinska Institute
Stockholm, Sweden

The Faculty of Pharmacy uses the Urkund system (plagiarism recognition) to examine all doctoral dissertations.

ISBN 978-951-51-6074-4 (Paperback)
ISBN 978-951-51-6075-1 (Online, http://ethesis.helsinki.fi)
ISSN 2342-3161 (Paperback)
ISSN 2342-317X (Online)

Painosalama Oy
Turku 2020

TABLE OF CONTENTS

ABSTRACT

Neurotrophic factor glial cell line-derived neurotrophic factor (GDNF), its co-receptor GDNF family receptor alpha 1 (GFRa1), and signaling receptor RET tyrosine kinase are essential to enteric nervous system (ENS) development; mice knockout for *Gdnf*, *Gfra1* or *Ret* lack the whole ENS distal to the stomach. These *Gdnf/Gfra1/Ret* knockout mice die at birth because of lack of ENS and kidneys hindering analysis of postnatal function of those proteins. Transgenic overexpression in animal models on the other hand relates to loss of physiological spatiotemporal regulation of gene expression. These two bottlenecks have hindered the understanding of the role and therapeutic potential of GDNF/GFRa1/RET signaling in congenital diseases, such as Hirschsprung's disease, and degenerative neurological diseases, such as Parkinson's disease. To tackle at least some of these problems, we have generated and characterized new mouse models with either increased or decreased gene expression dose - from the gene's endogenous locus and limited to naturally expressing cells.

Novel mouse models with increased expression were generated by editing 3' untranslated region (3'UTR) of the *Gdnf* gene in such a way that the edited 3'UTR lacks binding sites for negative regulators such as microRNAs. By preventing the posttranscriptional downregulation via the 3'UTR we were able to achieve *Gdnf* overexpression from the endogenous locus limited to the naturally *Gdnf* expressing cells. We showed that 3'UTR replacement or 3'UTR editing results in increased GDNF levels in the brain and kidneys, maintaining the spatiotemporal expression pattern with positive effects on the dopaminergic system and negative effects on the kidney size and urogenital tract development. We also found that 3'UTR regulates GDNF levels in the gastrointestinal tract and that 3'UTR controlled GDNF levels determine proportions of neuronal subtypes in the ENS. More specifically, inactivation of negative *Gdnf* 3'UTR regulation enhances nitrergic and cholinergic neuron numbers, and leads to increased gastrointestinal transit time, increased stool pellet size, and increased stool water content.

In congenital Hirschsprung's disease (HSCR) patients, on the other hand, lack of ENS ganglia in the distal gut leads to constipation and megacolon. Even though *RET* mutations are the most common cause of Hirschsprung's disease, no causative mutations in *GFRa1* are known. However, one study reported low *GFRa1* mRNA levels in some HSCR patients, suggesting that perhaps instead of being caused by mutations some HSCR cases could be triggered by reduced GFRa1 levels. Complicating the establishment of disease etiology in GDNF/GFRa1/RET related HSCR, postnatal viable HSCR mouse models with a defect in GDNF/GFRa1/RET signaling are not available. Here, we generated GFRa1 hypomorphic mice by insertion of a selectable marker gene in opposite transcriptional direction after the *Gfra1* exon 6. Insertion of an expression cassette in the opposite transcriptional direction often leads to under-expression from the other strand, resulting in hypomorph allele. We showed that a 70-80 % reduction in GFRa1 levels in mice resulted in congenital Hirschsprung's disease and associated enterocolitis phenotype with 100 % penetrance. We were also able to shed light in the chronology of events in the pathogenesis of Hirschsprung's disease associated enterocolitis: first goblet cell dysplasia accompanied by an

abnormal mucin phenotype is proceeding into epithelial damage, later followed by microbial enterocyte adherence and bacterial tissue invasion which likely leads to death by sepsis. Previously all those features had been described in patients but the sequence of events had remained unclear.

Our results suggest that dysregulation of *GDNF* or *GFRα1* levels by epigenetic mechanisms may play a role in normal and pathogenic development of the enteric nervous system.

TIIVISTELMÄ

Gliasolulinjaperäinen hermokasvutekijä (GDNF), sen ligandia sitova reseptori, GDNF perheen reseptori alfa 1 (GFRa1), ja signaalin välittävä reseptori RET-tyrosiinikinaasi ovat kaikki välttämättömiä ruuansulatuskanavan enteerisen hermoston kehittymiselle. *Gdnf*-, *Gfra1*- tai *Ret*-poistogeenisilta hiiriltä puuttuu koko enteerinen hermosto mahalaukusta nähden distaalisesti. Näiden poistogeenisten hiirten enteeristä hermostoa ei siis pystytä tutkimaan syntymän jälkeen, koska poikaset eivät ole elinkykyisiä puuttuvien munuaisten ja enteerisen hermoston takia. Lisäksi transgeeniseen yliekspressioon liittyy ongelmia, jotka johtuvat spatiotemporaalisen geenin ilmentymisen säätelyn puuttumisesta. Edellä mainitut kaksi eläinmallien yleistä ongelmaa ovat haitanneet GDNF/GFRa1/RET viestinvälityksen tutkimista synnynnäisten sairauksien, kuten Hirschsprungin taudin, ja hermorappeumasairauksien, kuten Parkinsonin taudin, tutkimuksessa. Aiempiin eläinmalleihin liittyvien ongelmien ratkaisemiseksi olemme kehittäneet ja karakterisoineet uusia hiirimalleja, jotka joko yli- tai ali-ilmentävät tutkittavaa geeniä endogeenisestä lokuksesta, jolloin geenin ilmentyminen rajoittuu sitä luontaisesti tuottaviin soluihin.

Geeniä yli-ilmentävät uudet hiirimallit tuotettiin muokkaamalla *Gdnf* geenin 3'-ei-transloitua aluetta (3'UTR) siten, että geenin ilmentymistä vähentävät tekijät, kuten mikroRNA:t, eivät voi enää siihen sitoutua. Kun transkription jälkeistä negatiivista säätelyä estettiin, pystyttiin GDNF:n yli-ilmentyminen rajoittamaan sitä luontaisesti tuottaviin soluihin. Osoitimme 3'UTR:n korvaamisen tai sen muokkaamisen johtavan lisääntyneeseen GDNF-tasoon aivoissa ja munuaisissa siten, että spatiotemporaalinen ilmentyminen säilyy vastaavana kuin villin tyypin hiirillä. Suuremmalla GDNF-tasolla on positiivisia vaikutuksia aivojen dopaminergiseen järjestelmään ja negatiivisia vaikutuksia munuaisten ja lisääntymiselinten kehittymiseen. Lisäksi havaitsimme, että 3'UTR säätelee GDNF-tasoa ruuansulatuskanavassa ja vaikuttaa enteerisen hermoston hermosolutyyppien suhteisiin. Tarkemmin sanottuna 3'UTR:n kautta tapahtuvan negatiivisen säätelyn estäminen lisää etenkin typpioksidia mutta myös asetyylikoliinia välittäjäaineena käyttävän hermotuksen määrää ja vaikuttaa luultavasti sitä kautta ruuansulatuskanavan toimintaan hidastaen suolen läpikulkuaikaa, suurentaen ulostepellettien kokoa ja lisäten ulosteen vesipitoisuutta.

Geeniä ali-ilmentävää eläinmallia käytettiin mallintamaan synnynnäistä Hirschsprungin tautia. Hirschsprungin tautia sairastavilta potilailta puuttuvat suoliston loppuosan enteerisen hermoston gangliot. Tämä aiheuttaa ummetusta ja johtaa paksusuolen laajentumiseen, megakooloniin. Vaikka *RET*-mutaatiot ovat yleisimpiä Hirschsprungin taudin aiheuttajia, ei tällaisia tautia aiheuttavia mutaatioita ole löydetty *GFRa1*-geenistä, vaikkakin yhdessä tutkimuksessa on raportoitu Hirscsprungin taudin potilailla vähentyneestä *GFRa1*-mRNA-tasosta. Tämä viittaa siihen, että mutaatioiden sijaan vähentynyt GFRa1:n määrä voi osalla potilaista osaltaan vaikuttaa Hirschsprungin taudin patogeneesiin. GDNF/GFRa1/RET-signaloinnin roolin tutkimista Hirschsprungin taudissa on vaikeuttanut elinkykyisten eläinmallien puuttuminen. Tässä tutkimuksessa kehitettiin GFRa1-hypomorfinen hiirimalli siten, että *Gfra1* geenin 6. eksonin jälkeen sijoitettiin selektiivinen markkerigeeni vastakkaiseen suuntaan transkriptioon nähden. Ekspressiokasetin lisääminen tällä tavalla johtaa usein vähentyneeseen ilmentymiseen toisesta juosteesta eli hypomorfiseen alleeliin. Osoitimme, että 70-80 % vähennys GFRa1-tasossa aiheuttaa hiirille 100 % penetranssilla fenotyypin, joka vastaa synnynnäistä Hirschsprungin

tautia ja siihen liittyvää enterokoliittia. Pystyimme myös selvittämään Hirschsprungin tautiin liittyvän enterokoliitin patogeneesin aikajärjestystä: ensin pikarisolujen dysplasia, johon liittyvät epänormaalit musiinit, sitten etenevä epiteelivaurio joiden jälkeen mikrobit voivat päästä kiinnittymään enterosyytteihin ja etenemään kudokseen, joka taas voi aiheuttaa sepsiksen ja kuoleman. Nämä kaikki on kuvattu potilailla, mutta tähän asti järjestys on ollut epäselvä.

Tulostemme perusteella *GDNF* tai *GFRα1* geenien ilmentymisen epigeneettinen säätely voi liittyä sekä enteerisen hermoston normaaliin että tauteihin liittyvään kehittymiseen.

LIST OF ORIGINAL PUBLICATIONS

I Anmol Kumar*, Jaakko Kopra*, Kärt Varendi*, **L. Lauriina Porokuokka**, Anne Panhelainen, Satu Kuure, Pepin Marshall, Nina Karalija, Mari-Anne Härma, Carolina Vilenius, Kersti Lilleväli, Triin Tekko, Jelena Mijatovic, Nita Pulkkinen, Madis Jacobsson, Maili Jakobsson, Roxana Ola, Erik Palm, Maria Lindahl, Ingrid Strömberg, Vootele Võikar, T. Petteri Piepponen, Mart Saarma*, Jaan-Olle Andressoo*: GDNF Overexpression from the Native Locus Reveals its Role in the Nigrostriatal Dopaminergic System Function. PLoS Genet 2015;11(12):e1005710 * equal contribution

II **L. Lauriina Porokuokka**, Heikki T. Virtanen, Jere Lindén, Yulia Sidorova, Tatiana Danilova, Maria Lindahl, Mart Saarma, Jaan-Olle Andressoo: Reduction in GDNF receptor Gfra1 levels results in long-segment Hirschsprung's disease and associated enterocolitis in mice. Cell Mol Gastroenterol Hepatol 2019;7(3):655-78

III **L. Lauriina Porokuokka**, Heikki T. Virtanen, Soophie Olfat, Richard Forsgård, Elin Org, Daniel Garton, Riitta Korpela, Jaan-Olle Andressoo: 3'UTR controlled GDNF levels regulate enteric nervous system development and adult gastrointestinal function in mice. Manuscript

The publications are referred in the text by their roman numerals. Reprints were made with the permission of copyright holders.

ABREVIATIONS

AChE	acetylcholinesterase
ARE	adenylate-uridylate rich elements
BMP	bone morphogenetic protein
CNS	central nervous system
DAT	dopamine transporter
EGC	enteric glial cell
ENCC	enteric neural crest derived cell
ENS	enteric nervous system
ERK	extracellular signal regulated kinase
ES	embryonic stem
FGF	fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GFRa1	Glial cell line-derived neurotrophic factor family receptor alpha-1
GI	Gastrointestinal
GPI	glycosylphosphatidylinositol
HAEC	Hirschsprung's disease associated enterocolitis
HSCR	Hirschsprung's disease
IND-B	Intestinal neuronal dysplasia B
KU	Knock-up
MAPK	mitogen-activated protein kinase
MEN	multiple endocrine neoplasia
MRE	microRNA binding site
NADPH-d	nicotinamide adenine dinucleotide phosphate diaphorase
NCAM	neural cell adhesion molecule
NOS	nitric oxide synthase
PD	Parkinson's disease
PGP9.5	protein gene product 9.5
PTEN	phosphatase and tensin homolog
RET	re-arranged during transfection, a tyrosine kinase
SOX10	SRY box–containing gene 10
TH	tyrosine hydroxylase
TUJ1	neuron-specific class III beta-tubulin
UTR	untranslated region

1. INTRODUCTION

Animal models are critical for enhancing understanding on gene function. Animal models with modified genes are also instrumental for the development and improvement of therapeutic treatments for human diseases (Doyle et al., 2012). While about 10.000 human diseases have monogenic origin, the majority of cases are influenced, at least to some degree, by the environment. The use of animal models is often required to define genetic contributions and to validate cellular and molecular pathways underlying these diseases, (Doyle et al., 2012), which allows treatment design strategy development. The number of genes is similar between high and low organisms. Instead, the complexity of an organism is in proportion to the non-coding RNA, emphasizing the role of spatiotemporal gene expression as well as expression level regulation (Levine and Tjian, 2003). While for example knockout animal models have been and still are useful in many study settings, there is a growing need for modeling increased or more subtle decreases in gene expression.

Neurotrophic factors are small, secreted proteins that support neuronal survival, differentiation, function, and maintenance in development and adulthood. Glial cell line-derived neurotrophic factor (GDNF), its co-receptor GDNF family receptor alpha-1 (GFRa1), and transmembrane signaling receptor tyrosine kinase, RET, are essential to enteric nervous system (ENS) development: mice knock-out for *Gdnf*, *Gfra1* or *Ret* are unviable due to lack of enteric ganglia and kidneys (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto Hideki, 1998). GDNF signaling through GFRa1 and RET has pleiotrophic effects on the enteric nervous system development, including chemoattraction in directed migration, proliferation, survival, and differentiation (Young et al., 1998; Worley et al., 2000; Gianino et al., 2003; Heanue and Pachnis, 2007; Uesaka et al., 2008; Uesaka et al., 2013). Previous attempts to study the effects of elevated GDNF expression levels in ENS development using random integration of transgenes with recombinant promoters or recombinant protein injections have been challenging, with problems arising such as wrong expression site or cell type, non-physiological expression levels, and wrong developmental timing (Wang et al., 2010; Mwizerwa et al., 2011; Doyle et al., 2012). The dearth of studies with postnatally viable conditional knockout animal models and the above fundamental technical issues with transgenic overexpression have hindered the understanding of the *in vivo* role of GDNF/GFRa1/RET signaling in ENS development and diseases. Besides the crucial role in the ENS development, GDNF is also known for its potent ability to promote survival of central nervous system (CNS) midbrain dopaminergic neurons (Lin et al., 1993; Hoffer et al., 1994), but despite this, newborn GDNF knockout mice as well as adult GDNF conditional knockout mice have an intact brain dopaminergic system (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Kopra et al., 2015). To further elaborate our understanding of GDNF's role, both in the ENS and in the CNS, we have, as one objective of this thesis, generated and analyzed new mouse models with increased GDNF levels from the endogenous locus.

Gene expression levels are regulated in numerous ways and each step in gene expression is not only extremely complex, but also regulated in a well-orchestrated manner. One of the key regulatory areas is 3' untranslated region (3'UTR) which takes part in mostly negative post-transcriptional regulation of gene expression (Mayr, 2017). Here, we generated novel mouse models with replaced or edited *Gdnf* 3'UTRs with a reduced number of binding sites for negative regulators, such as microRNAs. We showed that *Gdnf* 3'UTR replacement results in increased GDNF expression from the endogenous locus and characterized how 3'UTR mediated gene up-regulation of GDNF affects the nigrostriatal dopaminergic system (I) and the development and function of enteric nervous system (III). In section I, we showed that 3'UTR replacement results in increased *Gdnf* levels in the brain maintaining the striatal spatiotemporal expression pattern with positive effects on the dopaminergic system function. In section III, we showed that 3'UTR also regulates GDNF levels in the gastrointestinal tract and that the increased GDNF levels resulted in hyperganglionosis, changes in proportions of neuronal subtypes favoring nitrergic neurons, and changes in gastrointestinal function.

Hirschsprung's disease (HSCR) is a congenital malformation where ENS ganglia are missing from the distal gut, leading to severe constipation and megacolon (reviewed by (Heuckeroth, 2018)). Even though *RET* mutations are the most common cause of HSCR, no causative mutations in *GFRa1* are currently known (Butler Tjaden and Trainor, 2013; Goldstein et al., 2013), but in one study low GFRa1 levels have been measured in some HSCR patients with semi-quantitative tools (Lui et al., 2002). There have been no postnatal viable mouse models of HSCR with a defect in GDNF/GFRa1/RET signaling, shifting the focus of HSCR research to HSCR mouse models of endothelin-3 signaling pathway, where mouse models are post-natally viable. However, mutations in endothelin-3 signaling pathway account for only approximately 5 % of the patient cases (Kenny et al., 2010; Bondurand and Southard-Smith, 2016). Because about 50% of HSCR cases stem from alterations in RET, viable HSCR mouse models in this signaling pathway would allow comparative studies. To generate GFRa1 hypomorphic mice we utilized insertion of a selectable marker into the gene in the opposite transcriptional direction, often resulting in a hypomorphic allele (Meyers et al., 1998; Nagy et al., 1998; Wu et al., 2005; Wei et al., 2010; Wang et al., 2011). We showed that 70-80 % reduction in GFRa1 levels resulted in a congenital Hirschsprung's disease and associated enterocolitis (HAEC) phenotype with 100 % penetrance in GFRa1 hypomorphic mice. With this model system we were able to shed light into chronology of pathogenic events in HAEC, showing that goblet cell dysplasia is an early ubiquitous event, followed by increased goblet cell proliferation, mucus retention and accumulating epithelial damage providing an environment for later bacterial infection.

The mouse models presented in this thesis open further details on how GDNF/GFRa1/RET signaling regulates ENS development, composition and adult function and highlight the potential of epigenetic mechanisms in controlling GDNF/GFRa1 levels as potential disease drivers in congenital ENS disorders.

2. REVIEW OF THE LITERATURE

2.1 MOUSE MODELS

The house mouse (*Mus musculus*) was the first genetically modified animal model (Jaenisch and Mintz, 1974). There are numerous reasons as to why mice have become the most common model organism of human diseases: phylogenetic relatedness and physiological similarity to humans, ease and cost-effectiviness of maintaining and breeding mice, availability of strains and methods to develop new genetically modified animals (reviewed in (Doyle et al., 2012; Gurumurthy and Lloyd, 2019)). There is 99 % homology of genes between mice and humans (Waterston et al., 2002). In the early days, animal research was largely based on spontaneous mutations, like spontaneous dominant negative *Sox10* mutation in dominant megacolon mice and spontaneus gene deletion of *Ednrb* in piebald lethal mice. These were both used as models of Hirschsprung's disease (Webster, 1974; Hosoda et al., 1994; Southard-Smith et al., 1998). As incidence of spontaneous mutations is infrequent, mutations can be induced by random mutagenesis with for example radiation or ethylnitrosourea. However, nowadays reverse genetics approaches like directed gene recombination are most often used (Doyle et al., 2012).

2.1.1. REVERSE GENETICS METHODS

In gene targeting, modified DNA is introduced to embryonic stem cells and there by homologous recombination added to nuclear DNA (reviewed in (Gurumurthy and Lloyd, 2019)). Selectable marker genes that give an ability to grow in presence of selective agents normally toxic to cells, such as puromycin or neomycin cassettes, giving resistance to these antibiotics, are used to separate the transfected mouse embryonic stem (ES) cells. The correctly targeted ES cells are injected to blastocysts, thereby incorporating the modified cells into the transgenic animal and transferred to pseudopregnant females. Mice resulting from this approach are most often chimeras and obtaining germline transmission is needed to establish a founder line. Conditional approaches, such as bacteriophage cyclization recombination (Cre) recombinase between pairs of loxP sites (Cre/loxP) (Gu et al., 1994) or flippase and flippase recognition target (Flp/FRT) (Lakso et al., 1992), can be used for excision/recombination to physically remove selected sequences after transformation. Cre and Flp are both members of the λ integrase superfamily of site specific recombinases and share a common mechanism of DNA recombination that involves strand cleavage, exchange, and ligation (Sadowski, 1995). The conditional allele may be inactivated by Cre-mediated recombination, which can be restricted in time or space by crossing to different Cre-lines or viral-vector injections encoding for Cre.

However, gene targeting approaches are inefficient and slow compared to genome editing with programmable endonucleases, especially Clustered regularly interspaced short palindromic repeats and associated Cas proteins (CRISPR-Cas), which is taking over animal model design this decade (Cong et al., 2013; Lander, 2016). Programmable endonucleases can be used for genome editing because of their sequence-specific nuclease activity, which enables cleaving DNA at a

specific site. Why programmable endonucleases, such as Cas9 or Fok1, result in efficient genome editing relates to their ability to produce a double-stranded break at any desired location (reviewed in (Jasin and Rothstein, 2013)). RNA-guided Cas9 nuclease system has several advantages, such as simplicity, price, and efficiency, compared to the ES-cell-based-gene-targeting methods and previous non-CRISPR nuclease based platforms (Gurumurthy and Lloyd, 2019). Despite all its advantages, the RNA-guided Cas9 nuclease system also poses challenges, such as mosaicism (Yen et al., 2014) and off-target effects when applied directly on zygotes (reviewed in (Zhang et al., 2015)).

2.1.2 MOUSE MODELS WITH REDUCED GENE EXPRESSION

One of the most common approaches in genetics has been to disrupt the gene expression by knocking out the gene with targeted deletion. A consortium to systematically knockout every gene of mouse genome has been set and thousands of different knockout mice have been generated and phenotyped ((Hall et al., 2009; Guan et al., 2010) data available in www.kompphenotype.org). However, there are several limitation for the knockout approach. First of all, around 15 % of the knockout mice are embryonic lethal, preventing the use of knockout approach for many genes essential for cell viability (Hall et al., 2009). Second, the interpretation of the phenotype can be challenged with complex secondary, tertiary and even further effects resulting as a consequence of ablation of a pleiotrophic gene (Doyle et al., 2012). A third limitation is the ability of a living organisms to maintain the viability and fitness despite genetic variations, a quality known as robustness. Genetic robustness may arise from functionally redundant genetic pathways in developmental processes: as reported from several gene modified model organisms, the loss of one gene can be compensated by another with similar expression pattern and overlapping functions (Tautz, 1992; El-Brolosy and Stainier, 2017). Redundancy in gene families makes absence of a phenotype in a knockout model more likely than those for orphan genes (Barbaric et al., 2007). Robustness may also arise from tightly regulated metabolic, signaling and transcriptional networks where loss of a certain gene's functions may alter the expression of the other genes in that particular network (El-Brolosy and Stainier, 2017).

An improvement to the full knockout approach has been the conditional knockout, where the gene is conditionally knocked out only from certain tissues or at certain developmental time point restricted in time or space. With conditional ablation, the embryonic lethality may be avoided and some of the conditional knockout animals present a phenotype when the full knockout does not (El-Brolosy and Stainier, 2017). However, compensation by other genes may still mask effects of the studied gene (Barbaric et al., 2007).

As an alternative to full gene ablation in homozygous knockout animals, heterozygous mutant mice with half the gene dose, resulting in reduced function, can provide models with more relevance to the genetics of many human disorders, which often do not involve complete loss-of-function mutations (Kalueff et al., 2007). The embryonic lethality can be most often avoided in heterozygous mouse models, but for many genes the 50% reduction in gene activity is sufficient to maintain normal or close to normal function. To reduce the gene dose even further, a hypomorphic model may be generated with the same methods used for gene targeting (Baker, 2011). The generation of hypomorphic alleles is not always straight forward. For example,

insertion of a selectable marker cassette, such as neomycin or puromycin resistance cassette driven by strong promoter, often from cytomegalovirus (CMV) within an intron, may result in downregulated expression, a hypomorphic allele, or even in complete inactivation (Meyers et al., 1998; Nagy et al., 1998; Wolpowitz et al., 2000). A newer approach, such as adding consecutive adenosine nucleotides, polyA tracks, to the gene coding sequence in order to decrease translation elongation efficiency may also be used to achieve a hypomorphic allele (Arthur et al., 2017). While both knockout and hypomorph approaches are useful, many human diseases are instead characterized by gene overexpression (Prelich, 2012). Different approaches are needed to study these conditions.

2.1.3 MOUSE MODELS WITH INCREASED GENE EXPRESSION

Transgenic strains of mice are generated by the introduction of exogenous genes or DNA sequences (transgenes) that typically integrate as a single chromosomal insertion event. Random integration mutants of constitutive transgene expression in mice are informative, but they often do not accurately reflect what occurs in disease states (Doyle et al., 2012). The main problems related to transgenic overexpression are: 1) expression levels are too high, even hundreds to thousand fold of endogenous expression levels (Vogler et al., 2003; Shi et al., 2012), 2) loss of cell type specific expression pattern (Wang et al., 2010), and 3) loss of temporal control in expression (Ray et al., 1997; Wang et al., 2010). To combat loss of temporal control, approaches with drugs, such as doxycycline or tamoxifen, have been generated to better mimic the endogenous expression where the gene is turned on and off when needed. For the loss of cell type specific expression, the gene of interest is often expressed under the control of its native promoter but in a mutant background (Doyle et al., 2012). However, these improvements are insufficient to mimic endogenous expression pattern.

As the full knock out of a gene in an animal model often results in embryonic lethality or compensation confounding interpretation of the phenotype (El-Brolosy and Stainier, 2017) and disrupted spatiotemporal expression pattern and high expression levels related to transgenic approaches (Doyle et al., 2012), one option to study the effects of genes would be to instead alter the gene expression levels from the endogenous locus by addressing gene expression regulation.

2.2 GENE EXPRESSION LEVEL REGULATION

For eukaryotes, differences in gene expression between cell types are determined by expression of different sets of transcription regulators (reviewed in (Lelli et al., 2012). This is needed to form complex organisms and to respond to environmental stimuli. By default, eukaryote genes are in an “off-state”, needing to be switched on for gene expression. Gene expression level regulation is an extremely complex process. Even after the initiation of transcription, each step of gene expression can be modulated. Such steps include transcription, RNA processing, RNA transport, translation, and post-translational modification. One regulator can often control another regulator in a gene regulatory network (for reviews see (Levine and Davidson, 2005; Spitz and Duboule, 2008; Lee and Young, 2013)). *Trans*-regulatory elements are genes, such as transcription

factors, that modulate the expression of other genes from a distance through intermolecular interactions, hence "acting in *trans*" (for review see (Lambert et al., 2018)). *Cis*-acting regulatory elements are adjacent to the gene they regulate by interactions between different parts of the molecule hence "acting in *cis*" (For review see (Wittkopp and Kalay, 2011).

Besides the genetic code imprinted in DNA, epigenetic changes which regulate gene expression can also be heritable sometimes, but DNA methylation is influenced also, for instance, by sex and life experience (Allis and Jenuwein, 2016; Grimm et al., 2019). In response to environmental stimuli, the interplay between epigenetic modifications on DNA and histones is used for plasticity in cells that share identical genomes. This is done by providing a means of activating or silencing genes to affect gene expression of specific cell types (Smith and Meissner, 2013). Epigenetic changes cover epigenetic status of the chromatin, including DNA methylation, histone modifications, promoter–enhancer interactions, and noncoding RNA–mediated regulation. DNA methylation has been found at practically all regions of the genome, but promoter methylation is the best characterized. Promoter DNA methylation, typically occurring within CpG islands, results in powerful repression of transcription, primarily by recruiting repressor proteins or chromatin modifiers that enhance the binding to histones (Tate and Bird, 1993; Jones et al., 1998). CpG islands are short interspersed DNA sequences that deviate significantly from the average genomic pattern by being rich in GC and CpG (5'-C-phosphate-G-3'), and predominantly non-methylated (Deaton and Bird, 2011). In gene promoter regions the methylation of CpG islands is associated with loss of gene expression, which is common in development, differentiation, and cancer. In a genome wide single-cell dataset of DNA methylation in human embryonic tissue, the 3'UTR was recently shown to contain the highest DNA methylation levels compared to other non-coding regions, and this was linked to transcribed genes by correlation analysis (Luo et al., 2018). In patients, 3'UTRs have been shown to be subject to epigenetic up-regulation of gene-expression through DNA methylation. For example in colorectal cancer methylation in CpG islands of 3'UTR exons is positively associated with expression of IPF1/PDX1 and OTX1, both of which are homeodomain genes with important roles in development and are not expressed in normal colon (Smith et al., 2007). Recently many T-Cell activation related genes were also associated with upregulation via 3'UTR methylation in several types of human tumor tissue analysis, which could be linked to why T cells become "exhausted" and no longer target cancer cells (McGuire et al., 2019). Methylation in *Havcr2* 3'UTR resulted in overexpression of the gene in mice. Future studies will show how 3'UTR methylation regulates gene expression.

Tissue specific gene expression is driven by both transcription factors and *cis*-regulatory DNA sequences. Mutations affecting the activity of *cis*-regulatory sequences, instead of the transcription factors, are thought to be the most prevalent cause of phenotypic or morphological divergence (Wittkopp and Kalay, 2011). This makes non coding sequences, such as 3'UTRs, an interesting locus for designing animal models with altered gene expression levels.

2.2.1 3’ UNTRANSLATED REGION

mRNA has a tripartite structure consisting of a 5’ untranslated region, a coding sequence and a 3' untranslated region (3’UTR) (Figure 2.1). Human 3’UTRs are twice as long as other mammals’ 3’UTRs and about 10 times longer than in worms (Mayr, 2016). This information together with occurrence of alternative cleavage and polyadenylation to generate 3’UTR isoforms in more than half of the human genes, reflects the complexity of gene expression regulation by 3’UTRs (Mayr, 2016, 2017). Interestingly, many genes, especially in the CNS, bear exceptionally long 3’UTRs (>10 kb), specifically expressed in certain brain areas (Miura et al., 2013). 3’UTRs have *cis*-acting evolutionarily conserved sequences important for post-transcriptional gene expression regulation (Siepel et al., 2005; Xie et al., 2005). These *cis*-acting regulatory elements are sequences within the untranslated regions, introns or coding regions of precursor RNAs, as well as mRNAs which can be selectively recognized by a complementary set of *trans*-acting factors to regulate posttranscriptional gene expression. 3' UTR cis-elements are usually repeated and often act synergistically. Two key groups of conserved *cis*-acting elements in regulating gene expression levels via 3’UTRs are AU-rich elements (ARE) and microRNA responsive elements (MREs).

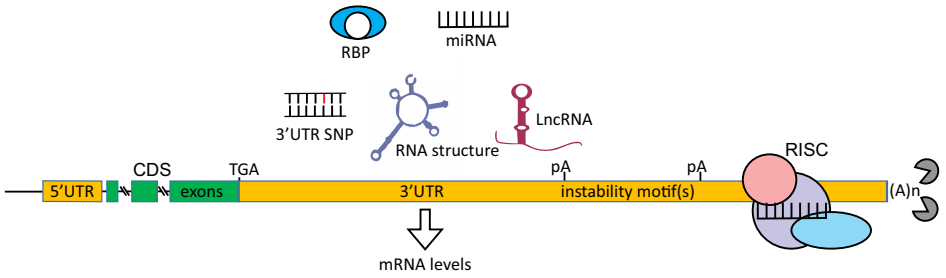

Figure 2.1. Simplified schema of the posttranscriptional regulation via the 3'UTR. mRNA stability and gene expression are regulated by various posttranscriptional modulators interacting with the 3'UTR. MicroRNAs (miRNAs) recruit the RNA-induced silencing complex (RISC) to specific target regions, leading to mRNA decay mediated by RNases. Instability motifs located within the 3' UTR are targeted by RBPs, resulting in rapid poly(A) tail deadenylation and mRNA degradation or stabilization. Single nucleotide polymorphisms (SNPs) in the 3'UTR disrupt the nucleotide complementarity needed for miRNA–mRNA interactions, altering the binding capacity of miRNAs. SNPs can also change the overall mRNA structure or particular instability motifs required for efficient RBP–mRNA interactions. Long non-coding RNAs (lncRNAs) are modifiers of miRNA and RBP activity through their sequestration, thereby suppressing their function. Finally, shortening of the 3'UTR through usage of alternative polyadenylation sites (pA) affects overall mRNA stability by decreasing the number of potential interaction sites/motifs for the previously mentioned posttranscriptional modulators. These regulatory elements acting in concert define the posttranscriptional regulome of the 3'UTR and ultimately the mRNA turnover and expression of a given gene. RBP, RNA binding protein, miRNA microRNA, CDS coding sequence. Modified from (Schwerk and Savan, 2015).

3' UTRs contain adenylate-uridylate-rich elements (AU-rich elements, ARE) that regulate mRNA stability. ARE, defined as regions containing frequent repeated adenine and uridine base patterns, are common in 3'UTRs and intronic regions of mammalian pre-mRNAs and tend to overlap with RNA-binding protein binding sites (Bakheet et al., 2018; Otsuka et al., 2019). AU-rich elements are particularly common in UTRs of proto-oncogenes, transcription factors and growth factors (Caput et al., 1986). RNA binding proteins via ARE binding can then either stabilize mRNA, like Hu antigens, or destabilize mRNA, like well-known RNA binding proteins tristetraprolin and AUF1 (Chen et al., 2001). mRNA degradation directed by ARE can be influenced in *trans* by exogenous factors like cytokines and transcription inhibitors (Kontoyiannis et al., 1999).

MicroRNAs are a class of single-stranded non-coding RNA molecules with an average 22 nucleotides that primarily interact with mRNA targets post-transcriptionally to negatively regulate expression as a part of epigenetic machinery (reviewed by (Piletič and Kunej, 2016; O'Brien et al., 2018)). Typically microRNAs are transcribed first to primary microRNAs, are then processed into precursor microRNAs and finally to mature microRNAs either through canonical or non-canonical pathways (O'Brien et al., 2018). The first discovered microRNA, lin-4, was found to regulate temporal development by targeting multiple sites in 3'UTR of *lin-14* gene in *Caenorhabditis elegans* (Wightman et al., 1993). However, in mammals any given microRNA alone has only a subtle effect on its target and microRNAs rather fine tune the gene expression levels in concert (Bartel, 2009). MicroRNA binding sites, MREs, comprise almost half of the conserved motifs in the 3'UTRs (Xie et al., 2005) and human protein coding genes have maintained their microRNA pairing in evolution in more than 60 % of the genes (Friedman et al., 2009). As expected, considering the shortness of binding region called "seed-sequence" (usually 6-8 bp), microRNAs are considered multivalent, which means that one microRNA can target several hundred genes and regulate several proteins in dynamic manner dependent on factors like cell-type or subcellular location, abundancy, target mRNA, and microRNA-mRNA interaction affinity (O'Brien et al., 2018). Factors predicting higher regulatory power of MREs are, for example, a position in a weaker secondary structure (for instance the ends of 3'UTR), complementary 3' binding of nucleotides 12-17, and two MREs close to each other (Grimson et al., 2007). MicroRNAs interacts most often with 3'UTR of target mRNA, reducing protein levels mainly by inducing mRNA degradation and but also translational repression (Garneau et al., 2007). However, interaction with 5'UTR, coding sequence and promoters is also possible (Lee et al., 2009). In more rare cases microRNAs can positively-regulate gene transcription by targeting promoter elements (Majid et al., 2010; Matsui et al., 2013). Deviant microRNA expression has been linked to a variety of human diseases such as cardiovascular diseases (for review see (Wojciechowska et al., 2017)), cancer (for review see (Leva et al., 2014)), diabetes (for review see (Guay et al., 2011)) and neurodegenerative disorders (for review see (Sharma and Lu, 2018)).

Both the addition and removal of the poly(A) tail are rate-limiting steps of maturation and degradation processes that the majority of mammalian mRNAs undergo. Around 70 % of human protein coding genes contain at least one alternative poly-adenylation signal in their 3'UTR sequence which results in alternative 3'UTRs in mRNAs (Derti et al., 2012; Lianoglou et al., 2013). The poly(A) tail as such may also have an effect on the fate of the transcript and it contains binding sites for poly(A) binding proteins and thus can affect mRNA export, stability and

decay (reviewed by (Jalkanen et al., 2014; Tian and Manley, 2017)). Also more than half of the mouse genes generate mRNA isoforms with alternative 3'UTRs encoding proteins with identical sequences (Hoque et al., 2013; Gruber et al., 2016). Alternative 3' UTR isoform ratios are tissue and cell type specific and change upon activation of signaling pathways during normal development and differentiation (Lianoglou et al., 2013; Gruber et al., 2016; Brumbaugh et al., 2018; Freimer et al., 2018). The evolution and roles of alternative 3'UTRs are still not well understood and for some reason are not intensively investigated. However, alternative 3'UTRs have been suggested to affect mRNA localization, mRNA stability and translation efficiency, as well as affecting protein functional diversity (Mayr, 2016). Further adding to this complexity, it has been recently suggested that the 3'UTR could also mediate protein localization via regions containing ELAVL1 binding sites (Berkovits and Mayr, 2015; Gruber et al., 2016).

One of the key problems of transgenic overexpressing animal models has been the loss of spatiotemporal expression pattern after random integration into the genome (Doyle et al., 2012). While negative regulation of gene expression through microRNAs is the best established role of the 3'UTRs (Oliveto et al., 2017), preventing this inhibition can be hypothesized to result in increased mRNA levels. When 3'UTR is genetically modified the gene expression pattern should also be retained in the naturally expressing cells, because 3'UTR regulation happens when mRNA has already been transcribed and when other parts of the gene regulatory orchestra have already taken care of the spatiotemporal controlling of expression. This makes 3'UTR mediated gene expression level regulation such a tempting candidate for increasing gene expression levels in the right cells at the right time.

2.2.1.1 MODELING 3'UTR REGULATION IN MICE

3'UTR regulation of gene expression has previously been studied in a handful of transgenic mouse models. The first model of 3'UTR mediated overexpression described was *c-fos* transgenic mice (Ruther et al., 1987). In these animals a high mRNA level could be measured from all tissues, when *c-fos* 3'UTR was replaced with a retroviral long terminal repeat. Thus 3'UTR mediated repression was necessary to c-fos level regulation (Ruther et al., 1987). The phenotype resulted from deletion of 3'UTR *cis* elements and overexpression through more efficient mRNA processing. In another study, the 3'UTR substitution approach was tested in ES cells and two mouse models. The gene modifications in mice resulted in a hypermorph model when *At1ar* 3'UTR was replaced with bovine growth hormone 3'UTR and in a hypomorph model when *c-fos* ARE was added to peroxisome proliferator-activated receptor γ 3'UTR (Kakoki et al., 2004). In a third study, transgenic mice carrying a 3'UTR-truncated *Hmga2* cDNA overexpressed high mobility group AT-hook 2 (HMGA2) resulting in proliferative hematopoiesis (Ikeda et al., 2011). Recently, methylation of the 3'UTR of an immune checkpoint gene linked to T cell activation in cancer, *Havcr2*, resulted in overexpression of this gene in mice (McGuire et al., 2019). Treating activated T cells with the demethylating agent decitabine, or knocking out the DNA methylating enzyme Dnmt3a in mice resulted in decreased 3'UTR methylation and gene expression of *Havcr2*. Therefore, the authors suggested that 3'UTR may serve as a functionally relevant site of DNA methylation.

3'UTR replacement does not result in increased gene expression and unaffected expression pattern of all studied genes. When a 3'UTR with alternative short and long forms is replaced with a single poly-(A) signal, normal cellular mRNA localization can be lost. This is seen in the *Camk2a* mouse model (Miller et al., 2002; Mayr, 2018) and *Bdnf* mouse model (An et al., 2008), with both cases leading to impaired memory. Mice with only short *Bdnf* 3'UTR also become obese, a phenotype that could be rescued with an injection of a virus encoding *Bdnf* with long 3'UTR (Liao et al., 2012). This indicates that the long *Bdnf* 3'UTR plays a role in energy balance (Liao et al., 2012).

The results from mice with altered 3'UTRs show that gene expression levels can be decreased and increased by 3'UTR replacement or even via methylation of 3'UTR. The data suggests that 3'UTRs can affect subcellular localization, however, in this situation the expression is still retained in the naturally expressing cell types. For *GDNF* no 3'UTR mediated localization signals have been reported. Analysis of RNA sequencing data from neurites versus cell soma of motor-neurons has suggested lack of preferential enrichment between these compartments (Rotem et al., 2017; Nijssen et al., 2018). Our unpublished data has supported these findings. We cannot however, exclude effects in other cell types or under different culturing conditions.

2.3 NEUROTROPHIC FACTORS AND THEIR RECEPTORS

According to the classical neurotrophic factor hypothesis, developing neurons compete for trophic support (Hamburger and Levi-Montalcini, 1949). During development, a substantial portion of central and peripheral nervous system neurons undergo programmed cell death when lacking or not obtaining this support in sufficient amounts (Dekkers et al., 2013). The first characterized neurotrophic factor, nerve growth factor, was extracted from mouse sarcoma in the 1950'es by the later Nobel Laureates Rita Levi-Montalcini and Stanley Cohen (Levi-Montalcini and Cohen, 1956; Cohen and Levi-Montalcini, 1957). Based on the ligand structure, neurotrophic factors can be divided to three major groups: neurotrophins (reviewed in (Saragovi et al., 2019)), GDNF family ligands (reviewed in (Airaksinen and Saarma, 2002; Kim and Kim, 2018)), and neurokines (reviewed in (Nathanson, 2012)). However, also an unconventional neurotrophic factor family of proteins CDNF and MANF has been described (reviewed in (Lindahl et al., 2017)). Neurotrophic factors are small, secreted proteins that support neuronal survival, differentiation, function, and maintenance in development and adulthood. Despite the names, neurotrophic factors are also important for other cell types and developmental processes. For example, mice lacking the potent survival factor for dopaminergic neurons, GDNF, are not viable due to lacking kidneys and enteric nervous system, but have an intact dopaminergic innervation at birth (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). GDNF can be argued to be an interesting candidate for studies including altering gene expression levels as GDNF has long and conserved 3'UTR and which plays an important role in several developmental processes as well as in neuronal survival.

2.3.1 GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR

GDNF signaling together with co-receptor GFRa1 through transmembrane signaling receptor RET is an ancient signaling system conserved in vertebrates, including fish, birds and mammals (Hearn et al., 1998; Shepherd et al., 2001). The proteins are so similar that zebrafish GDNF and GFRa1 can activate human RET and promote survival of mouse dopaminergic neurons *in vitro* (Saarenpaa et al., 2017). Though GDNF family ligands are not found in flies, GFRa and RET have orthologues in *Drosophila melanogaster* (Kallijarvi et al., 2012). Originally GDNF, distant member of transforming growth factor beta superfamily, was found from an immortalized glial cell line as a factor promoting dopaminergic cell survival *in vitro* (Lin et al., 1993). GDNF/GFRa1/RET signaling is essential in the development of ENS and kidneys, but is also important in the development of teeth and eye innervation (Hashino et al., 2001; de Vicente et al., 2002). GDNF also promotes the survival or function of many neuronal types, including the midbrain dopaminergic neurons and motor neurons (Lin et al., 1993; Henderson et al., 1994; Kopra et al., 2017). Besides GDNF/GFRa1/RET signaling also has an essential role in spermatogenesis (Naughton et al., 2006) and is needed for renewal of many other types of stem cells (Xiao et al., 2014; Peng et al., 2017; Perea et al., 2017).

Classically GDNF family ligands GDNF, neurturin, artemin, and persephin were found to signal trough transmembrane signaling receptor tyrosine kinase (RET), encoded by ret proto-oncogene, after first binding to corresponding glycosylphosphatidylinositol (GPI) anchored co-receptor GDNF family receptor alpha-1 (GFRa1), GFRa2, GFRa3 or GFRa4 (Figure 2.2) (Airaksinen and Saarma, 2002; Kim and Kim, 2018). As well as GFRa1, GDNF also has weaker affinity for GFRa2 and GFRa3. GDNF may also signal through alternative receptors like neural cell adhesion molecule 1 (neurite outgrowth or Schwann cell migration (Paratcha et al., 2003; Nielsen et al., 2009)), syndecan 3 independent of GFRa1 (neurite outgrowth (Bespalov et al., 2011)) and neuropilin 1 (in glioma progression (Sun et al., 2017)). Binding of homodimeric GDNF and GFRa1 to RET stimulates receptor dimerization (Figure 2.2, (Airaksinen and Saarma, 2002)) and activation of downstream signaling pathways, like mitogen-activated protein kinase (MAPK)/ extracellular signal regulated kinase (ERK) (Natarajan et al., 2002), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) (Besset et al., 2000), Janus kinase/signal transducer and activator of transcription (Plaza-Menacho et al., 2007) or Src family kinases (Popsueva et al., 2003). These signaling pathways are needed in processes such as cell differentiation, growth, migration, self-renewal of embryonic stem cells and survival.

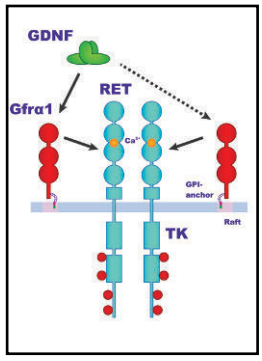

Figure 2.2. GDND/GFRa1/RET signaling. GDNF activates RET tyrosine kinase by first binding to GFRa1-receptor that is attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor and is predicted to have three globular cysteine-rich domains. (Modified from (Airaksinen and Saarma, 2002)). TK tyrosine kinase.

Although first characterized as neurotrophic factor secreted from glial cells *in vitro*, GDNF has been shown *in vivo* to be also secreted from the developing gut (Hellmich et al., 1996; Natarajan et al., 2002) and kidney mesenchyme (Durbec et al., 1996; Pichel et al.,

1996), testicular sertoli cells (Trupp et al., 1995; Hellmich et al., 1996; Meng et al., 2000), and striatal parvalbuminergic neurons (Hidalgo-Figueroa et al., 2012). GDNF expression from glial cells is evident *in vitro* from central and enteric nervous system derived glial cells (U87 cells, C6 cells, enteric glial cells (Verity et al., 1998; Verity et al., 1999; Le Berre-Scoul et al., 2017)) and also *in vivo* from gliomas (Wiesenhofer et al., 2000). The abnormal GDNF expression in gliomas results from epigenetic modifications in sequence elements unrelated to genetic mutations like hypermethylation of GDNF promoter and histone modifications (reviewed in (Ayanlaja et al., 2018)). Mouse and human *GDNF* 3'UTRs are conserved and contain several putative binding sites for RNA binding proteins (Oh-hashi et al., 2012) and microRNAs (MirTarBase). Whether GDNF levels are affected by 3'UTR deletion or replacement has not been studied previously.

GFRa1 was first discovered using expression cloning to isolate cell surface GDNF-binding proteins (Jing et al., 1996; Treanor et al., 1996). It is a cell surface receptor proteolytically processed from preprotein to form a mature receptor for GDNF and with lesser affinity to structurally related neurturin (Cik et al., 2000). GFRa1 is expressed by several different types of neurons and glial cells in both the CNS, PNS, and ENS (Trupp et al., 1997; Yu et al., 1998). Besides the membrane bound GPI-anchored form, GFRa1 exists also in a free-floating, soluble form. Activation of GPI-anchored GFRa1 induces cis-signaling within the cell to which it is bound, whereas extracellular soluble form of GFRa1 may trans-signal upon neighboring cells. GFRa1 may act as an ligand-induced cell adhesion molecule where GDNF induces trans-homophilic binding between GFRa1 molecules and cell-adhesion between GFRa1 expressing cells in synaptogenesis (Ledda et al., 2007). The role of GDNF/GFRa1/RET signaling in ENS development and disease is discussed further in the sections 2.5 and 2.6.

2.4 GDNF IN THE CENTRAL NERVOUS SYSTEM

In the CNS, GDNF expression peak is at P15 (Hidalgo-Figueroa et al., 2012). GDNF is expressed in numerous brain structures including the striatum, nucleus accumbens, septum, thalamus and cerebellum (Trupp et al., 1997; Golden et al., 1998; Hidalgo-Figueroa et al., 2012). In the adult murine brain GDNF is expressed by scattered medium sized neurons throughout the striatum (Trupp et al., 1997). Of these GDNF expressing neurons, 95 % have been shown to be parvalbumin positive (Hidalgo-Figueroa et al., 2012). As well as playing a role in the dopaminergic system elaborated in the next chapter, GDNF has also a survival effect on spinal cord motor neurons and a role in the axonal growth or guidance and synapse formation of hippocampal neurons, cortical neurons, and spinal cord motor neurons in the CNS (Paratcha and Ledda, 2008).

Besides being a potent neurotrophic factor *in vitro* for the mesencephalic dopaminergic neurons (Lin et al., 1993) GDNF also promotes dopaminergic neuronal survival and neurorestoration of adult brains post-injury in rodent and primate animal models (Hoffer et al., 1994; Gash et al., 1996). This makes GDNF a potential therapeutic candidate for Parkinson's disease (PD). PD is a chronic, progressive neurodegenerative disorder characterized by rigidity, bradykinesia and tremor resulting from death of dopaminergic neurons in *substantia nigra* giving rise to lack of dopamine in the striatum (for a recent review see (Del Rey et al., 2018)). GDNF has been studied in several clinical trials of PD. As proteins do not penetrate the blood-brain-barrier, GDNF treatment

cannot be given orally. Instead the protein needs to be administered directly to the action site in the brain. Clinical studies using different administration methods to give GDNF directly to the brain have resulted in inconsistent outcomes. Intracerebroventricular delivery of GDNF has failed in phase I trials and resulted in a substantial amount of side effects at the same time (Kordower et al., 1999; Nutt et al., 2003). The lack of effects with the intracerebroventricular dosing of GDNF has been explained by GDNF not reaching the target tissues: the striatum and substantia nigra (Kordower et al., 1999; Nutt et al., 2003). In later studies, GDNF was given directly to the striatum. Promising results were obtained from Phase I studies where GDNF was administered to the striatum (Gill et al., 2003; Slevin et al., 2007) but a randomized phase II study was interrupted because of lack of effects (Lang et al., 2006). This was likely as a result of an ineffective pump system being unable to distribute GDNF evenly (Salvatore et al., 2006; Morrison et al., 2007). The newest clinical trials treating PD with GDNF give some hope for restoring the dopaminergic phenotype of some cell bodies when given as an infusion to the putamen in every 4 weeks but motor improvements were seen only in the open label study (Whone et al., 2019a; Whone et al., 2019b).

Interestingly, mice knockout for GDNF, GFRa1 or RET have intact dopaminergic system at birth (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto Hideki, 1998) but, as mentioned previously, these mice are unviable because of congenital defects in the kidneys and ENS. This makes it impossible to study postnatal maturation of the dopaminergic system. In 2008, it was suggested by Pascual et al. that GDNF is required to maintain the adult dopaminergic system (Pascual et al., 2008). In the study by Pascual et al. one *Gdnf* allele was knocked out and the other was "floxed". Esr1-Cre was then used for the adult full knockout of *Gdnf* via a high dose of tamoxifen at 2 months of age. The mice presented a PD phenotype seven months later, with hypokinesia and decreased dopamine synthesis rate limiting enzyme tyrosine hydroxylase (TH). However, these results were challenged by a later study, where GDNF did not affect the number or survival of catecholaminergic neurons in three different *Gdnf* conditional knockout mouse models (Kopra et al., 2015). First, *Gdnf* deletion with Nestin Cre from the CNS around embryonic day 10 did not have an effect on the dopaminergic system. The lack of effects in the Nestin Cre conditional *Gdnf* knockout could possibly be explained by compensation by other trophic factors, a phenomenon often associated with developmental models. In second and third approach, *Gdnf* was deleted from adult animals either locally, using intrastriatal unilateral AAV Cre delivery, or by repeating the experimental protocol published in the study by Pascual et al. 2008. The authors reported spontaneous deletion of *Gdnf* between 2-5 months of age independent of tamoxifen application in Esr1-Cre mouse line. This showed that Esr1-Cre induces recombination also without the presence of tamoxifen. There was no effect in the number, survival or function of the catecholaminergic neurons in the two adult knockout models nor in the developmental deletion model. The three complementary approaches showed that GDNF reduction or deletion did not lead to any significant changes in the number of central monoaminergic neurons. However, rather than controlling dopamine neuron numbers, endogenous GDNF in the striatum regulates the function of dopamine neurons. In a later study with adult onset striatal AAV-Cre delivery *Gdnf* conditional knockout mice were shown to display reduced spontaneous and amphetamine induced locomotor activity and striatal dopamine efflux (Kopra et al., 2017). In the same study embryonic *Gdnf* deletion with Nestin Cre did not affect striatal dopamine levels or

dopamine release, but dopamine reuptake was increased due to increased levels of both total and synaptic membrane-associated dopamine transporters (DAT) (Kopra et al., 2017). Hence, *Gdnf* deletion seems to have an effect on the dopamine system function in these mouse models. Activation of endogenous GDNF production therefore still has interesting therapeutic potential for Parkinson's disease and should be further explored.

2.5 ENTERIC NERVOUS SYSTEM

At the end of the 19th century, Englishmen William M. Bayliss and Ernest H. Starling performed a historical experiment in dogs, where they induced pressure to intestinal lumen resulting in oral contraction and anal relaxation allowing production of a propulsive wave: nowadays called peristaltic reflex (Bayliss and Starling, 1899). This reflex persisted even after extrinsic nerves were severed and the enteric nervous system (ENS) was specified as a self-contained hub independent of CNS input. This was confirmed year 1917 *in vitro*, as peristaltic reflex remains in isolated gut (Trendelenburg, 2006). Besides peristalsis, ENS regulates secretions, blood supply and immune responses in the gastrointestinal (GI) tract.

Due to its structural complexity and ability to remain functional without the CNS, ENS is often called "the brain in the gut" or "the second brain". Many features of the ENS resemble the CNS more than other parts of peripheric nervous system: enteric glia resembling astrocytes, no collagen in the ganglia, interneurons, microcircuits, a small extracellular space, dense synaptic neuropil, isolation from blood vessels, multiple synaptic mechanisms and multiple neurotransmitters (Gershon, 1999; Goldstein et al., 2013). Evolutionally the ENS should be considered "the first brain", because this kind of nervous system, consisting of plexuses of intrinsic neurons of the gut wall and innervation controlling muscle movements, is found in all animals that have neurons, including hydra and echinoderms, which do not possess CNS, that in turn is deduced to be bilaterian development (Furness and Stebbing, 2018). Neurochemical similarities across phyla imply a common origin of the ENS from hydra to humans (Furness and Stebbing, 2018). Besides the ENS, the GI tract is innervated by the CNS controlled extrinsic nervous system consisting of sympathetic and parasympathetic pathways. The afferent and efferent network between CNS and ENS assures bi-directional message transport between these systems (parasympathetic Vagus & pelvic nerves and sympathetic pathways). Interestingly 90 % of vagal afferents between ENS and CNS seem to go from the gut to the brain (Furness et al., 2014).

In mammals the ENS covers the GI tract from the esophagus to the anal sphincter, as a network of ganglia distributed to two distinct plexuses: the myenteric plexus in the whole GI tract and the submucosal plexus in the intestine. The myenteric plexus is located between the longitudinal and circular layers of *muscularis externa* and is the main neuronal regulator of intestinal motor function. The main function of the submucosal plexus located within the connective tissue of the submucosa is to control for GI secretion and local blood flow. The ENS projects to the pancreas, gallbladder, trachea and prevertebral ganglia. Virtually every class of neurotransmitter found in the CNS has also been detected in the ENS (Table 2.1, (Costa et al., 1996; Furness et al., 2014). The ENS in the small intestine and colon contains full reflex circuits, including sensory neurons, interneurons and several classes of motor neurons, through which gastrointestinal functions like

muscle activity, transmucosal fluid fluxes and local blood flow are controlled. Based on marker gene expression, morphology, location, and projection targets, approximately ten distinct cell types have been described in the myenteric plexus (Furness and Stebbing, 2018; Zeisel et al., 2018) and four in the submucosal plexus of the mouse (Bornstein, 2008; Furness and Stebbing, 2018). Based on recent single cell RNA sequencing data, enteric neurons can be roughly split into nitrergic and cholinergic groups based on *Nos1* and *Chat/Slc5a7* expression respectively, where calretinin is a marker for some of the cholinergic subpopulations (Zeisel et al., 2018). Considering the different tasks of the esophagus, stomach, small and large intestine it is evident that there are differences in the ENS of these regions. There are also differences between for example the different regions of the colon where the proximal colon has more complex ENS circuits(Li et al., 2019b). Most of the transcription factors linked to the ENS development have been found to be conserved between mouse and humans (Memic et al., 2018). However, in the mature ENS there are some differences even between two rodent species, mice and guinea pigs (Gabella and Trigg, 1984).

Table 2.1. Neuronal subtypes of the small intestine ENS (combined from (Bornstein, 2008; Furness, 2012)). MP myenteric plexus, SMP submucosal plexus, EXTR external innervation, ChAT choline acetyltransferase, TK tachykinin, IR immunoreactive, ENK enkephalin, NOS nitric oxide synthase, VIP vasoactive intestine peptide, 5-HT serotonin, SOM somatostatin, GABA γ-aminobutyric acid, PACAP pituitary adenylyl-cyclase-activating peptide, NPY neuropeptide Y

Basic Function	**Plexus**	**Neurotransmitters and neurochemical markers**
Intrinsic sensory neuron, AH/Dogiel type II	MP	ChAT/calbindin/TK-IR
Ascending interneuron	MP	ChAT/calretinin/TK/ENK
Descending interneuron	MP	ChAT/5-HT, targets myenteric and/or submucosal ganglia
Descending interneuron	MP	ChAT/SOM, targets myenteric and/or submucosal ganglia
Descending interneuron	MP	Ach/ATP/NOS/VIP
Excitatory longitudinal muscle motor neuron	MP	ChAT/calretinin/TK
Excitatory circular-muscle motor neuron	MP	ChAT/TK, short or long oral projection
Cholinergic secretomotor neuron	MP	ChAT/NPY/Opioid peptides, CCK, GRP
Intestinofugal neurons to sympathetic ganglia	MP	ChAT/VIP/
Inhibitory longitudinal muscle motor neuron	MP	NOS/VIP/GABA, rare in guinea-pig ileum
Inhibitory circular-muscle motor neuron	MP	NOS/VIP/PACAP/ENK, short anal projection
Inhibitory circular-muscle motor neuron	MP	NOS/VIP/PACAP/GRP, long anal projection
Descending interneuron	MP	NOS/VIP/GRP/±ChAT
Noncholinergic secretomotor neuron	MP	VIP
Intrinsic sensory neuron, AH/Dogiel type II	SMP	ChAT/TK-IR
Cholinergic secretomotor neuron	SMP	ChAT/NPY
Noncholinergic secretomotor neuron	SMP	VIP/PACAP/NPY
Vasodilator neuron	SMP	ChAT/calretinin, cholinergic
Sympathetic neurons, secretion inhibiting	EXTR	Noradrenaline
Sympathetic neurons, motility inhibiting	EXTR	Noradrenaline
Sympathetic neurons, vasoconstrictor	EXTR	Noradrenaline, ATP

2.5.1 ENTERIC NERVOUS SYSTEM DEVELOPMENT AND MAINTENANCE AND GDNF/GFRA1/RET

The neural crest is a key feature that separates vertebrates from other craniate organisms (Gans and Northcutt, 1983). During late gastrulation or early neurulation, the neural crest cell population arises at the border between the neural and non-neural ectoderm to delaminate from their tissue of origin and migrate to their specific locations (reviewed in (Bronner and Simões-Costa, 2016)). In the developing mouse gut, ENS progenitor cells termed enteric neural crest-derived cells (ENCCs), from the vagal neural crest somite levels 1-7 enter the foregut on embryonic day 9-9.5 (E9-E9.5; 4 weeks of gestation in humans, Figure 2.3) (reviewed in (McKeown et al., 2013; Obermayr et al., 2013)). After the relatively small number of precursors enters the foregut, ENCCs proliferate actively and in the end generate the millions of enteric neurons and glia covering the whole mature intestine (Gianino et al., 2003). ENCCs migrate rostro-caudally along the gut towards a GDNF source secreted from the mesenchyme, reach the proximal colon at E12, and colon colonization is complete by E14-E14.5 [7 weeks of gestation in humans (Young et al., 1998; Sasselli et al., 2012). ENS development is different from PNS and CNS development in the sense, that the massive programmed cell death does not occur which likely relates to fetal enteric microenvironment being especially rich in trophic support (Chalazonitis et al., 2012). In the developing gut GDNF is expressed in the intestinal mesenchyme, while GFRa1 is expressed both in the mesenchyme and ENCCs, and RET is expressed only in the ENCCs (Durbec et al., 1996; Young et al., 1998; Worley et al., 2000; Young et al., 2001; Lui et al., 2002; Natarajan et al., 2002). GDNF, GFRa1, and RET are essential to the enteric nervous system development: a mutation in an early-required factor such as GDNF, GFR1 or RET, causes aganglionosis because all lineages of neurons in the intestines arise from precursors that require RET stimulation (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto Hideki, 1998).

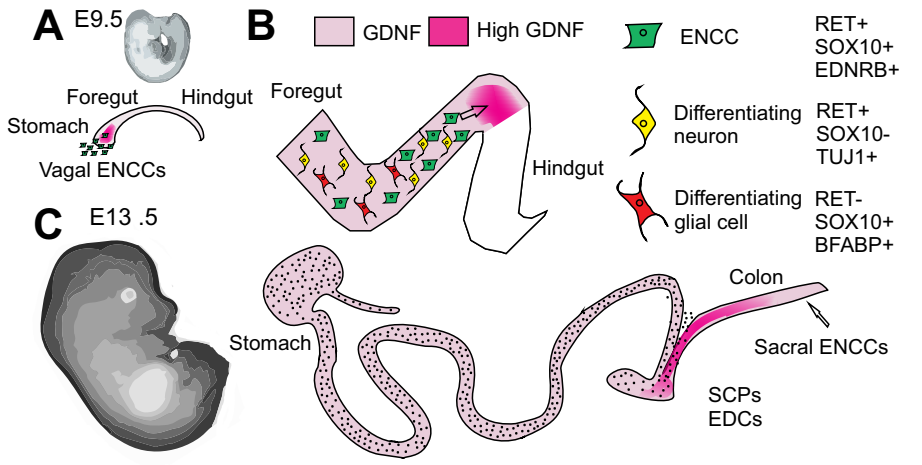

Figure 2.3. Mouse ENS development (modified from (Heanue and Pachnis, 2007))**.** A) The precursors for enteric neurons and glia termed enteric neural crest derived cells (ENCC) arising from the vagal neural crest enter mouse foregut at embryonic day 9.5 (E9.5). B) During the migration/ colonization process (approximately E9.5-E14.5) ENCCs are first following a chemoattractant GDNF gradient. Behind the wavefront ENCCs proliferate and start to differentiate to neuronal and glial lineages. C) By E13.5 the ENCC wavefront has reached approximately the mid colon. Besides to the vagal ENCCs, also Schwann cell precursors (SCPs), endoderm derived cells (EDCs), and sacral ENCCs contribute to the mature ENS. GDNF, GFRa1 and RET are essential to the ENS development affecting migration, proliferation and differentiation. SOX10 SRY box–containing gene 10, EDNRB endothelin receptor B, TUJ1 neuron-specific class III beta-tubulin, BFABP brain fatty acid binding protein.

In the hindgut ENCCs can migrate either through a trans-mesenteric pathway (Nishiyama et al., 2012) or along the caecum to populate the colon (Druckenbrod and Epstein, 2005). In addition, truncal and sacral neural crest derived cells contribute to the ENS development (reviewed by (Nagy and Goldstein, 2017)). According to Uesaka et al. (Uesaka et al., 2015) there is a 5-20 % subpopulation of ENS cells, mostly calretinin positive neurons, in the distal gut, arising from Schwann cell precursor lineage during early postnatal development in mice and invading the gut through extrinsic innervation. Knocking out *Ret* specifically from Schwann cell precursors resulted in a 30 % loss of ENS cells in the colon of studied mice (Uesaka et al., 2015). Based on another recent study on transgenic animals, a small contribution to the small intestine myenteric neurons also seems to come from the endoderm derived cells (Brokhman et al., 2019).

In addition to regulating ENCC migration, GDNF signaling controls the proliferation, differentiation and survival of ENCCs (Gianino et al., 2003; Heanue and Pachnis, 2007; Uesaka et al., 2008; Mwizerwa et al., 2011; Uesaka et al., 2013). The size of the proliferating pool of precursors is critically important for normal ENS development. There is a critical density of cells at the wavefront necessary to form the cellular strands that drive ENCC migration (Le Douarin and Teillet, 1973, 1974) and a low ENCC density delays the rate of migration (Pomeranz and Gershon,

1990; Gershon and Ratcliffe, 2004). Some ENCCs differentiate to neurons during the migration process, occurring earliest at E10.5-E11.5 in mice (Rothman and Gershon, 1982; Branchek and Gershon, 1989; Young et al., 2002). Neurogenesis continues through development and through the first weeks of postnatal period (Pham et al., 1991; Wang et al., 2010; Laranjeira et al., 2011; Uesaka et al., 2015). Precursors of different neuronal subtypes exit the cell cycle at different times (Pham et al., 1991). The intracellular signaling pathways PI3/AKT and MAPK/ERK were activated in avian enteric crest derived cells *in vitro* by GDNF and PI3/AKT was stimulating proliferation (Focke et al., 2001). There seems to be a developmental change as a function of age for GDNF from a mitogenic to a differentiation/survival response by the developing enteric neurons (Chalazonitis et al., 1998). Premature differentiation of ENCCs has been reported *in ovo*, which has been linked to both reduced and increased GDNF levels (Mwizerwa et al., 2011). The timing of this shift corresponded to developmental changes in gut expression of GFRa1, where the GFRa1 expression has been reported to become increasingly more localized between E13.5 and E19.5 and to be restricted to myenteric plexus by E19.5 (Worley et al., 2000). The membrane-associated GPI-anchored form and the extracellular soluble form of GFRa1 can function cooperatively in mediating GDNF signaling in ENCCs (Worley et al., 2000).

GDNF is needed throughout development and it has been reported to be also abundant in the adult gut (Peters et al., 1998), but its postnatal role is not well characterized. Quantitative PCR approach has revealed higher levels of *RET* (20x), *GFRα1* (2x), and *GFRα2* (2x) mRNA in human colonic myenteric ganglia compared to muscle layers, and higher levels of *GDNF* and *NRTN* expression located to circular and longitudinal muscle layers compared to myenteric plexus (Barrenschee et al., 2013). RET, GFRa1 and GFRa2 have also been shown to co-localize in myenteric and submucosal ganglia by immunohistochemistry in adult human colon by the same authors (Barrenschee et al., 2013). *In vitro* GDNF can also promote both structural and functional plasticity in primary cultures of rat postnatal myenteric neurons (Rodrigues et al., 2011). Here the authors suggested enriched neonatal intestinal smooth muscle cells immunoreactive for α-smooth muscle actin as a source for GDNF *in vitro*. Increased GDNF levels have been reported in inflammatory bowel disease (von Boyen et al., 2011; Steinkamp et al., 2012). GDNF is proposed to be expressed by enteric glial cells (EGCs) in inflammatory states and *in vitro* (von Boyen et al., 2011; Steinkamp et al., 2012; Le Berre-Scoul et al., 2017). However, detailed descriptions of GDNF expression in the adult gut are missing.

GDNF has been shown to have some therapeutic effects in several rodent models of dextran sulfate induced colitis (Zhang et al., 2010; Liu et al., 2014; Meir et al., 2019). In the first mouse model of colitis, intracolonic delivery of adenovirus carrying GDNF partly restored intestinal epithelial barrier function, which was measured as epithelial permeability and a decrease in inflammatory markers (Zhang et al., 2010). The authors suggested that GDNF mediates cross-talk between EGCs and mucosal epithelial cells. In the second model, intracolonic adenoviral GDNF delivery partly prevented dextran sulfate induced enteric neuron loss, reduced expression of some inflammatory markers (tumor necrosis factor and interleukin β) and improved delayed colonic transit in rats (Liu et al., 2014). In the third colitis model, recombinant human GDNF partly

alleviated inflammation-induced changes in the intestinal epithelial barrier (Meir et al., 2019). GDNF levels were also studied in inflammatory bowel disease patients. Decreased GDNF levels were measured by ELISA in samples from resected inflamed ileums of Crohn's disease patients and inflamed colons of ulcerative colitis patients (Meir et al., 2019).

Diverticular disease is a widespread disease in industrialized countries initially characterized by multiple mucosal or submucosal outpouchings throughout the colon which may lead to a broad spectrum of symptoms, typically constipation and flatus. Decreased neuronal density in both myenteric and submucosal plexus has been reported in patients with diverticular disease (Wedel et al., 2010). Decreased mRNA expression of *GDNF*, *GFRa1* and *RET* has also been reported (Böttner et al., 2013). The authors proposed that hypoganglionosis linked to diverticular disease could be due to the lack of neurotrophic support mediated by the GDNF system.

During the latest decade, studies have started to show that the neurogenesis continues in postnatal ENS. In a study by Laranjeira et al., locally applied detergent benzalkonium chloride was used to dose-dependently ablate the myenteric plexus of a small area of the small intestine of adult reporter mice (Laranjeira et al., 2011). They showed that in the damaged mouse gut SRY box–containing gene 10 (SOX10) positive neural crest cells generate both neuronal and glial lineages of enteric ganglia. Lineage tracing of SOX10 positive precursors showed that 2.8 % of the adult undamaged ENS were formed from precursors labeled at P7.5, 1.6 % generated from P30 progenitors but none from P84 progenitors, indicating that postnatal neurogenesis from SOX10 positive progenitors is limited after early postnatal stages. Microbiota seems to play an important role for the postnatal ENS neurogenesis, since germfree mice display alterations in the ENS, such as decrease in nerve density, a decrease in the number of neurons per ganglion and an increase in the proportion of myenteric nitrergic neurons(Collins et al., 2014). The changes in the ENS of adult germfree mouse gut may be normalized after microbial colonization (De Vadder et al., 2018). The authors reported maturation of neuronal precursors in the myenteric plexus of the colon after microbial transplantation and suggested that the mechanism for this is restoration of serotonin signaling through 5-HT4R receptors. The role for serotonin and 5-HT4R receptors is supported also by the findings in dextran sulfate sodium induced murine colitis model which resulted in increased numbers of myenteric neurons, and this was inhibited by 5-HT4 antagonism (Belkind-Gerson et al., 2015). The role of GDNF/GFRa1/RET signaling for postnatal neurogenesis has not been studied thoroughly.

To date the role GDNF/GFRa1/RET signaling in adult ENS function and maintenance is not clear and even the expressing cell type is controversial. Further studies are needed to address these matters and to dissect the role of GDNF/GFRa1/RET signaling for epithelial barrier function and inflammation.

2.6 ENS RELATED DISEASES AND GDNF/GFRa1/ RET SIGNALING

The importance of the ENS is emphasized by the life-threatening effects of enteric neuropathies. A lack of an ENS is life-threatening in most primitive animals that have a nervous system and in humans, who cannot survive the congenital lack of an ENS (Furness, 2012). Congenital ENS malformations resulting from disruption of NCC development can be classified as neurocristopathies, which covers a broad spectrum of congenital malformations. Primary developmental disorders of the enteric nervous system can be grouped to ones with abnormal number of neurons - aganglionosis, hyperganglionosis, and hypoganglionosis, and ones with abnormal differentiation of neurons resulting in changes in biochemical and physiological properties like ganglioneuromatosis (Kapur, 2000; Puri and Rolle, 2004). As further defined in this thesis, GDNF/GFRa1/RET signaling has a major role in ENS disorders, aganglionosis and hyperganglionosis.

2.6.1 HIRSCHSPRUNG'S DISEASE AND ASSOCIATED ENTEROCOLITIS

Congenital megacolon where enteric neurons are missing from the distal part of the gut was named after Danish pediatrician Harald Hirschsprung, who reported two cases of "Constipation in the newborn as a result of dilation and hypertrophy of the colon" in 1888 (Hirschsprung 1888, English translation (1981)). The first descriptions of Hirschsprung's disease dates earlier to 17th century to Dutch physician Fredrick Ruysch (Leenders and Sieber, 1970) and even earlier to ancient Hindu surgeons treating the condition with ayurvedic medicine (Raveenthiran, 2011).

Congenital intestinal aganglionosis is the most common form of congenital ENS malformations and affects about one in every 5000 babies, with a 4:1 males to females ratio (Butler Tjaden and Trainor, 2013). As a result of the lack of the enteric neurons in distal intestine, typically the rectosigmoid colon, the contents of the gut cannot pass normally. This results in functional intestinal obstruction, leading to constipation, gut distention (megacolon) and failure to thrive. The underlying pathogenic etiology is based on defects in the craniocaudal migration, proliferation, differentiation, and survival of ENCCs in the early stage of pregnancy (Amiel et al., 2008; Butler Tjaden and Trainor, 2013). HSCR is generally subdivided into short-segment, long-segment, and total colonic aganglionosis types based on the point at which histologically characterized aganglionosis begins (Ryan et al., 1992). The aganglionated section is surgically removed, but somewhat surprisingly, the patients remain at risk of Hirschsprung's disease associated enterocolitis (HAEC) even after the operation (Frykman and Short, 2012).

HSCR is a multigenic disease, with environmental factors affecting the disease prevalence. However, the most common genetic cause of HSCR is the presence of inactivating mutations in *RET*. These account for approximately 50% of familial cases and 15-35% sporadic cases (Plaza-Menacho et al., 2006; Amiel et al., 2008; Kenny et al., 2010; Butler Tjaden and Trainor, 2013). In addition to *RET* mutations, at least 13 other HSCR susceptibility genes have been identified, accounting for approximately 20 % of the cases (Plaza-Menacho et al., 2006; Kenny et al., 2010;

Ruiz-Ferrer et al., 2011; Butler Tjaden and Trainor, 2013; Goldstein et al., 2013). Even though *RET* is the most common genetic cause for HSCR, mutations in *GDNF* have been reported only in a couple of patients (Ivanchuk et al., 1996; Hofstra et al., 2000; Kenny et al., 2010) and no causative *GFRα1* mutations have been found. However, in one study, reduced levels of *GFRα1* in HSCR patients have been reported using only semiquantitative tools such as band intensity measurement on agarose from semi-quantitative PCR products (Lui et al., 2002). HSCR penetrance is clearly also influenced by gene expression levels since copy number variants of *MAPK10*, *ZFHX1B*, and *SOX2* loci were associated with HSCR in a pilot study on 67 candidate genes (Jiang et al., 2011). Currently, in approximately half of the sporadic cases of HSCR the underlying genetic cause remains unknown (Butler Tjaden and Trainor, 2013; Goldstein et al., 2013).

There is a relationship between 3'UTR *RET* variants and HSCR (reviewed in (Torroglosa et al., 2019)). Several *RET* polymorphisms were characterized in a group of HSCR patients and controls (Fitze et al., 2003). Two variants located at the 3'UTR were found (c.3187+47T>C (rs2075912) and 30UTR+124A>G)) which had a strong association with HSCR (Fitze et al., 2003). A protective *RET* haplotype has also been identified in the RET 3'UTR: a single nucleotide polymorphism g.128496T>C (rs3026785) (Griseri et al., 2007). Here the authors suggested, based on *in vitro* reporter gene expression, that the protective effect of this allele against HSCR might be due to lower mRNA degradation. This could lead to an increase of gene transcripts and hence an increase in the amount of total RET protein. Seven more haplotypes with negative association with HSCR were found in a screening of the *RET* 3'UTR in a Chinese population (Pan et al., 2012). The microRNAs associated with regulation of gene expression by these *RET* polymorphisms has not been elucidated. Epigenetic changes in *GDNF* and *GFRα1* genes have so far not been studied. However, by using different tools for bioinformatic analysis (ENSEMBL BioMart for sequences, EMBOSS CpGplot for PCpG island prediction) our collaborator has found a large CpG islands in GFRa1 promoter (Saara Ollila, unpublished findings).

After GDNF/GFRa1/RET signaling, endothelin 3 (EDN3), its receptor endothelin receptor B (EDNRB), and its biosynthetic endothelin converting enzyme (ECE1) together are the second most common genetic cause for HSCR, being responsible for about 5 % of HSCR cases (Kenny et al., 2010). However, it has remained controversial as to why EDN3 signaling gives rise to HSCR. Since many knockout mouse models of HSCR related genes, including RET, GFRa1 and, GDNF, result in total intestinal aganglionosis, HSCR animal research has concentrated on the endothelin-3 (EDN3) signaling pathway where the knockouts of *Edn3*, *Ednrb*, or *Ece1* lead to aganglionosis of the distal colon (Baynash et al., 1994; Hosoda et al., 1994; Yanagisawa et al., 1998). EDN3 is not needed for the formation of enteric neurons, like GDNF/GFRa1/RET signaling, but rather to prevent premature differentiation of neuronal precursors and to keep them in proliferative state (Hearn et al., 1998; Barlow et al., 2003; Nagy and Goldstein, 2006). EDN3 and GDNF signaling are working in a coordinated manner to enhance ENCC proliferation (Barlow et al., 2003). On the other hand, EDN3 opposes the GDNF migration and differentiation (Hearn et al., 1998; Barlow et al., 2003; Heuckeroth, 2003; Nagy and Goldstein, 2006). There is a need for viable animal models with defects in GDNF/GFRa1/RET signaling to counterbalance the bias in HSCR studies towards EDN3/EDNRB/ECE. If and why this is important remains to be elucidated once appropriate animal models have been generated and fully analyzed. However, it is safe to assume that the best way

to mimic human condition is by modulating the causative molecular signaling cascade. As up to half of the HSCR cases result from alterations in RET, HSCR mouse models with dampened GDNF/GFRa1/RET signaling would be relevant for HSCR and related HAEC etiology studies. Next, such models would allow comparisons and genetics analysis of endothelin pathway in HSCR/HAEC.

HAEC is the leading cause of mortality in HSCR. It is characterized by altered mucin composition, mucin retention, bacterial adhesion to enterocytes, and epithelial damage, although the order of these events has been obscure. A recent hypothesis of HAEC pathogenesis includes luminal microbial alterations in conjunction with impaired mucosal barrier function and innate immune responses, allowing for the bacterial translocation and the development of HAEC (Gosain and Brinkman, 2015). However, the pathogenesis of HAEC remains poorly understood. HAEC develops in as many as every third patient with short-segment HSCR and every second patient with long-segment HSRC (Murphy and Puri, 2005; Frykman and Short, 2012). This suggests either multigenic and/or environmental contribution (Frykman and Short, 2012; Demehri et al., 2013). Clinical symptoms of HAEC include fever, abdominal distention, diarrhea and sepsis. HAEC is histopathologically characterized in the colon by crypt dilatation, mucin retention, enterocyte adherence of bacteria, a shift from acidic towards neutral mucin production, epithelial damage, leukocyte infiltration, ulceration and, in the terminal stages, transmural necrosis and perforation (Murphy and Puri, 2005; Frykman and Short, 2012; Demehri et al., 2013). As mentioned above, the sequence of events in HAEC progression is not clear, at least in part because lack of animal models genocopying congenital form of HSCR associated to defects in GDNF/GFRa1/RET signaling.

Mucins are produced primarily by goblet cells and form a protective barrier layer preventing bacterial enterocyte adherence (Pelaseyed et al., 2014). The inner colon mucus layer is rapidly renewed and converted into the outer mucus layer by controlled endogenous proteolytic (Johansson and Hansson, 2013). Decreased levels of the main protein component of these layers, the MUC2 mucin have been reported in stool samples from HSCR patients (Mattar et al., 2003). Goblet cell hyperplasia (Thiagarajah et al., 2014) and altered goblet cell function (Nakamura et al., 2018b) have been reported in HSCR. Altered goblet cell function was proposed to result in intestinal barrier dysfunction which would contribute to the development of HAEC (Nakamura et al., 2018b). HAEC and inflammatory bowel disease have similar clinical presentations including diarrhea, blood in the stools and abdominal pain (Nakamura et al., 2018a). According to a meta-analysis of HSCR patients, male patients with extensive colonic aganglionosis, who continue to suffer from postoperative HAEC post-operatively, are particularly more susceptible to developing inflammatory bowel disease (Nakamura et al., 2018a). As GDNF/GFRa1/RET signaling has been linked to HSCR and inflammation, further studies are needed to establish whether the defective GDNF/GFRa1/RET signaling makes HSCR patients more susceptible to inflammation.

2.6.1.1 MOUSE MODELS OF HIRSCHSPRUNG'S DISEASE

Mouse models of Hirschsprung's disease have been extensively reviewed by Bondurand and Southard-Smith (Bondurand and Southard-Smith, 2016). Of the HSCR susceptibility genes, a postnatally viable mouse model with colonic aganglionosis, has previously been reported for *Edn3, Ece, Ednrb, Sox10, Phox2b,* and *Ttf1*.

Studies using transgenic mice have shed light on how GDNF signaling regulates the development of the enteric nervous system (ENS). As already mentioned, mice that lack expression of genes encoding GDNF, GFRa1, or RET have a common phenotype that includes kidney agenesis and a lack of enteric ganglia distal to the stomach (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Enomoto Hideki, 1998). However, heterozygous *Gdnf, Gfra1* or *Ret* null-allele mice with a reduced gene dose, have a relatively mild reduction in enteric neuron numbers and do not develop the clinical features of childhood HSCR or HAEC (Gianino et al., 2003). Only when kept in an isogenic background 20 % of *Gdnf* knockout heterozygous mice develop megacolon because of hypoganglionosis (Shen et al., 2002) but this mouse model does not replicate human HSCR with distal aganglionosis (Table 2.2).

The RET gene is alternatively spliced at its 3' end to produce multiple isoforms, of which RET9 and RET51 are the most highly expressed (Tahira et al., 1990). In the intestine, like in most tissues, RET9 and RET51 are co-expressed, RET9 being more abundant there (de Graaff et al., 2001; Perea et al., 2017). Mice lacking RET9 isomer develop colonic aganglionosis accompanied with small and malformed kidneys, while mice lacking RET51 appear healthy (de Graaff et al., 2001). Ret9/-, with reduced RET and RET9 expression, have been shown to develop very short segment distal colonic aganglionosis with incomplete penetrance and no kidney agenesis (Uesaka et al., 2008). However, no postnatal data of megacolon in these mice has been reported. Mouse studies using knock-in or timed conditional deletion alleles for *Gfra1* or *Ret* support the importance of GFRa1/RET signaling in ENS development and survival through postnatal day 1 (P1) (de Graaff et al., 2001; Uesaka et al., 2007; Uesaka et al., 2008). This means that animal models with defective GDNF/GFRa1/RET signaling, that phenocopy postnatal HSCR and/or HAEC, have not previously been available. This has led to a bias in the basic research towards the endothelin 3 signaling pathway, since these animals are viable after birth (Table 2.2). For further research it is essentially important to produce viable HSCR mouse models with defects in GDNF/GFRa1/RET signaling because the majority of known HSCR mutations are in RET.

Table 2.2. Selected mouse models of Hirschsprung's disease for GDNF/GFRa1/RET and endothelin 3 signaling pathways (revised from (Bondurand and Southard-Smith, 2016)). ENS enteric nervous system, ENCC enteric neural crest derived cell, KO knockout, TIA total intestinal aganglionosis, HSCR Hirschsprung's disease.

	HSCR susceptibility gene	Role in ENS development	Expression site during ENS development	Mouse models	Developmental defect	Mature phenotype	Mouse model references	HSCR references
RET	Ret proto-oncogene	Required early factor for ENS development: survival, proliferation, differentiation, and migration	ENCCs and differentiating neurons, dynamic manner	$Ret^{tm1Cos/tm1Cos}$ ($Ret^{-/-}$), Targeted gene knock-out	Altered migration, proliferation, survival, and neuronal differentiation of ENCCs	KO mice TIA	(Schuchardt et al., 1994; de Graaff et al., 2001; Gianino et al., 2003)	(Attie et al., 1995)
				Ret hypomorphic isoforms, humanized monomeric isoform, multiple alleles that produce only one isoform or alter phosphorylation of the receptor including $Ret^{mi51/mi51}$, $Ret^{9/-}$, Ret^{S697A}	Decreased ENCCs, failure to migrate into fetal midgut, compromised neuronal survival. Depend on mutated isoform.	Colonic aganglionosis	(Schuchardt et al., 1994; de Graaff et al., 2001; Uesaka et al., 2008)	
				$Ret^{tm3(RET)/mi/+}$, targeted mutation of cytoplasm domain	Uncharacterized	Homozygous mice TIA, heterozygotes hypoganglionosis and reduced fiber density	(Jain et al., 2004)	
				$Ret^{tmCti/+}$ ($Ret^{C620R/+}$) targeted amino acid substitution	Uncharacterized	Homozygous mice TIA, heterozygotes hypoganglionosis and reduced fiber density	(Carniti et al., 2006)	
				$Ret^{tm3Cos/tm3Cos}$ ($Ret^{S697A/S697A}$) targeted nucleotide substitution	Delayed migration of ENCCs into colon	Aganglionosis of mid and distal colon	(Asai et al., 2006)	
GDNF	Glial cell line-derived neurotrophic factor	Survival, proliferation, differentiation, and migration	Mesenchyme, in non ENCCs	$Gdnf^{tm1Lmgd/tm1Lmgd}$ ($Gdnf^{-/-}$) targeted gene knockout	GDNF affects survival, proliferation, differentiation, and migration	KO mice TIA, hypoganglionosis in heterozygotes	(Pichel et al., 1996; Sanchez et al., 1996; Gianino et al., 2003)	(Angrist et al., 1996; Ivanchuk et al., 1996; Ruiz-Ferrer et al., 2011)
GFRα1	GDNF family receptor alpha-1	GDNF co-receptor for RET signaling	Differentiated neurons, ENCCs, and mesenchyme	$Gfra1^{tm1Jmi/tm1Jmi}$ ($Gfra1^{-/-}$) targeted gene knockout	Mediates GDNF signaling through RET	KO mice TIA, heterozygotes reduced neuron size	(Enomoto Hideki, 1998; Gianino et al., 2003)	(Lui et al., 2002)
NRTN	Neurturin	Size of mature enteric neurons and the extent of neuronal projections	Circular layer of the external smooth muscle layer	$Nrtn^{tm1Jmi/tm1Jmi}$	RET ligand (co-receptor GFRa2), promotes neurite outgrowth	KO mice have reduced neuron size, fiber density, and motility	(Heuckeroth et al., 1999)	(Doray et al., 1998; Ruiz-Ferrer et al., 2011)
EDN3	Endothelin 3	Prevent premature differentiation	Mesenchyme, in non ENCCs	$Edn3^{ls/ls}$ (Lethal Spotting)	Migrations of ENCCs	Aganglionosis in distal colon, decreased neuronal numbers	(Baynash et al., 1994)	(Hofstra et al., 1996)
				$Edn3^{tm1Ywa/tm1Ywa}$ targeted gene knockout ($Edn3^{-/-}$)	Uncharacterized	Aganglionosis in distal colon	(Baynash et al., 1994)	
EDNRB	Endothelin receptor type B	Receptor for endothelin 3	ENS precursors	$Ednrb^{s/s}$ (piebald) spontaneous insertion in intron, hypomorphic	Defective migration of ENCCs into the hindgut	Hypomorphic allele, rare colonic aganglionosis	(Hosoda et al., 1994; McCallion et al., 2003; Yamada et al., 2006)	(Puffenberger et al., 1994)
				Ednrbs-l/s-l (piebald lethal) spontaneous gene deletion	Uncharacterized	HSCR, decreased AChE fiber density in proximal intestine, alterations in neuron types in ganglionated regions of colon	(Webster, 1973; Fujimoto, 1988; Hosoda et al., 1994; Cantrell et al., 2004)	
				$Ednrb^{tm1Ywa/tm1Ywa}$ (Ednrb-/-) targeted gene knockout	Uncharacterized	HSCR, hypoganglionosis of proximal intestine	(Hosoda et al., 1994; Cantrell et al., 2004)	
ECE1	Endothelin-converting enzyme 1	Catalyzes the proteolytic activation of endothelin	Mesenchyme	$Ece1^{tm1Reh/tm1Reh}$ ($Ece1^{-/-}$) targeted gene knockout	Defective migration of ENCCs into the hindgut	HSCR	(Yanagisawa et al., 1998)	(Hofstra et al., 1999)

2.6.2 INTESTINAL NEURONAL HYPERGANGLIONOSIS

Hyperganglionosis is rarely reported, except for the transition zone between ganglionated and aganglionic gut parts in Hirschsprung's disease (Ure et al., 1994). However, in rare cases chronic constipation and/or pseudo-HSCR can be caused by hyperganglionosis.

Intestinal neuronal dysplasia type B (IND-B) is a condition defined by hyperganglionosis and hyperplasia of submucous plexus, but often includes myenteric hyperplasia. IND-B is commonly associated with childhood constipation, which sometimes leads to intestinal pseudo-obstruction and megacolon with a suggested incidence of 1:7500 (reviewed in (Toledo de Arruda Lourenção et al., 2016; Kapur and Reyes-Mugica, 2019)). The symptoms in IND-B patients include nausea, vomiting, abdominal distention, abdominal pain, and constipation or even total absence of motility. These are similar to symptoms caused by mechanical obstruction in the small intestine, such as a tumor or scar tissue. Even life-threatening chronic intestinal pseudo-obstruction has been described with associated complications, such as bacterial infections and poor absorption of nutrients. IND-B type of changes are often found in HSCR patients proximal to the transition zone (Ure et al., 1994; Kobayashi et al., 1995). There still remains controversy surrounding the diagnostic criteria of IND-B. One of the confounding factors in, that the size and distribution of the ganglia in control patients varies greatly and has not been thoroughly studied (Coerdt et al., 2004; Kapur and Reyes-Mugica, 2019). Colonic neuronal hyperplasia was first described by Meier-Ruge in 1971 in three patients with severe motility disturbances correlating with an increase in the number of ganglia both in myenteric and submucosal plexus (Meier-Ruge, 1971). Submucosal plexus hyperplasia and giant ganglia that may be 2-3 times the size of normal ganglia are the main diagnostic criteria; but inconsistency of these criteria has been in the spotlight when the existence of IND-B has been debated (Meier-Ruge et al., 2004; Kapur and Reyes-Mugica, 2019). In addition to ganglion size variation in controls, one of the key problems for IND-B diagnosis is the fact that submucosal hyperganglionosis may occur in healthy infants (Meier-Ruge et al., 2006). IND-B is treated with dietary adjustments, laxatives, and enemas with good results in 80 % of the patients (Schimpl et al., 2004). Surgery is an option to patients resistant to conservative treatment (Toledo de Arruda Lourenção et al., 2016; Kapur and Reyes-Mugica, 2019). In most of the patients, symptoms of IND-B can resolve by the age of 4 years because of the ENS maturation (Bruder and Meier-Ruge, 2007). In some patients, degeneration of enteric neurons has been described. In a chronic intestinal pseudo-obstruction patient case study, hyperganglionosis was found from dilated small intestine segment at the age of 5, but 9 years later the authors described hypoganglionosis (Di Nardo et al., 2006). The authors hypothesized that the neuronal hyperplasia is related to adaptive changes of the ENS in a response to the obstruction and that also the neuronal cell loss, likely by apoptosis, is later triggered by this obstruction.

Genetic causes of IND-B remain a mystery (Toledo de Arruda Lourenção et al., 2016; Kapur and Reyes-Mugica, 2019). Only RET polymorphism has been linked to IND-B (Fernandez et al., 2009) but as strong a link to IND-B genetics as to HSCR is currently missing. Some different RET polymorphisms in isolated IND-B patients, compared to HSCR patients with IND-B or healthy controls, have been found (Fernandez et al., 2009). The same study authors also found a R982C sequence variant present in three HSCR+IND-B cases and in one isolated IND-B case (Fernandez et al., 2009). This, interestingly, has initially been found in a family with both multiple endocrine

dysplasia A and HSCR but also with a mutation in codon 618, a typical finding in MEN2A (Mulligan et al., 1994). Although the molecular mechanisms associated with the morphological features of IND-B are somewhat unclear, knockout mouse models of negative regulators of GDNF/GFRa1/ RET signaling, namely *Spry2*$^{-/-}$ mice, display enteric neuronal hyperplasia (Taketomi et al., 2005). Hyperganglionosis with megacolon is observed in another negative regulator, *Kif26a,* deficient mice (Zhou et al., 2009). These mouse models are further discussed in the next chapter. Three main hypothesis have been made for the developmental pathogenesis of IND-B: (i) inhibition of normal cell death that occurs in the developing ENS after birth, (ii) excess proliferation of the enteric neuronal precursor cells or (iii) a secondary reaction of the ENS to compensate for a functional abnormality of the gut (Hatano et al., 1997a). When normal cell death is prevented by electroporating a dominant-negative form of caspase-9 into vagal NCCs in the chicken foregut, the resulting hyperganglionosis is suggested to be direct result of inhibiting normal cell death in ENS precursor cells (Wallace et al., 2009). This supports the first hypothesis. Taken together, both the genes and the mechanisms underlying IND-B pathogenesis remain to be discovered.

Another condition leading to hyperganglionosis is diffuse intestinal ganglioneuromatosis, which is an extremely rare disease with benign tumors of ganglionic cells leading to constipation and distention of the gut. 40-90 % of diffuse intestinal ganglioneuromatosis is associated with MEN2B (Gfroerer et al., 2017). The gastrointestinal symptoms include hypomotility, constipation, diarrhea, abnormal sphincter functions, bleeding and diverticula. These can lead to an intestinal pseudo-obstruction and the development of megacolon (Gfroerer et al., 2017; Castinetti et al., 2018; Kapur and Reyes-Mugica, 2019). Pathology findings include submucous and myenteric plexus hyperplasia and giant cholinergic ganglia (Feichter et al., 2009; Gfroerer et al., 2017). The severity of neural hyperplasia is usually more pronounced than in IND-B and may include grossly obvious tumors (Kapur and Reyes-Mugica, 2019). The onset of hyperplasia in ganglioneuromatosis is difficult to determine, but it is likely that the lesions keep growing postnatally, even in adulthood (Kapur and Reyes-Mugica, 2019). Intestinal ganglioneuromatosis has been reported sometimes in conjunction with IND-B (Al-Rikabi et al., 2011). In 95% of MEN2B cases, codon 918 of *RET* is mutated, leading to an exchange of methionine by threonine and leading to constitutive activation of RET (Gujral and Mulligan, 2006). Hyperganglionosis has not been reported in MEN2B mice (Smith-Hicks et al., 2000), but MEN2A mice, conditionally expressing human RET51 with C618F mutation, display enteric hyperganglionosis and C-cell hyperplasia without developing thyroid carcinoma (Okamoto et al., 2019). It has been suggested that the genetic alterations involved in ganglioneuromatosis could be linked to IND-B by analogy and investigation of these pathways including GDNF/GFRa1/RET would improve the understanding of IND-B pathogenesis and phenotype (Kapur and Reyes-Mugica, 2019).

2.6.2.1 MOUSE MODELS OF HYPERGANGLIONOSIS

Hyperganglionosis resulting in intestinal obstruction or chronic constipation, is rare, as are the mouse models of hyperganglionosis. Altogether eight different postnatal viable mouse models with hyperganglionosis have been described (Table 2.3). Two of these can be called intestinal neuronal dysplasia B models (Table 2.3, (Hatano et al., 1997a; Zhou et al., 2009)). In one of these, GDNF/GFRa1/RET signaling is indirectly affected by inactivation of negative regulators of the RET signaling pathway, Kinesin superfamily protein 26A (Kif26A). Another two can be categorized as

models of ganglioneuromatosis: mice with targeted MEN2A mutation constitutively activating RET and mice with a conditional knockout for *Pten* (Table 2.3, (Puig et al., 2009; Okamoto et al., 2019)). Four more miscellaneous hyperganglionic mice have been described, two of which involve GDNF/GFRa1/RET signaling (Table 2.3). In addition some embryonic lethal knockout mouse models with enteric hyperganglionosis accompanied with other congenital defects have been described including Sonic Hedgehog knockout mice (Ramalho-Santos et al., 2000), transcription factor *Zic2* knockout mice (Zhang and Niswander, 2013), and growth suppressor *Gas1* knockout mice (Biau et al., 2013; Jin et al., 2015). The postnatal viable mouse models are further described in the next chapter.

Table 2.3. Postnatal viable mouse models of hyperganglionosis (revised from (Bondurand and Southard-Smith, 2016)).

Gene		Role in ENS development	Animal model	Description	References
Intestinal neuronal dysplasia mouse models					
Tlx2 (Ncx/ Hox11L.1)	T cell leukemia, homeobox 2, Hox Transcription factor	Expressed in neural crest-derived tissue, cellular growth and differentiation	$Tlx2^{tm1Sjk\ /\ tm1Sjk}$, ($Ncx^{-/-}$) mice with targeted gene knockout	Intestinal neuronal dysplasia B: colonic hyperganglionosis, megacolon	(Hatano et al., 1997a; Yamataka et al., 2001; Parisi et al., 2003; Kato et al., 2009)
Kif26a	Kinesin family member 26A	Negative regulator of GDNF signaling *in vitro*	$Kif26a^{tm1.1Noh/\ tm1.1Noh}$ ($Kif26a^{-/-}$) targeted gene knockout mice	Intestinal neuronal dysplasia B: megacolon, enteric nerve hyperplasia, hypersensitivity for GDNF-Ret signaling	(Zhou et al., 2009)
Ganglionneuromatosis mouse models					
Ret	Ret proto-ongogene	Survival, proliferation, differentiation, and migration	$Ret^{51(C618F)/51(C618F)}$ mice with targeted human MEN2A mutation	Ganglionneuromatosis: hyperganglionosis of the intestine and C-cell hyperplasia without medullary thyroid carcinoma	(Okamoto et al., 2019)
Pten	Phosphatase and tensin homology	Inhibits migration and proliferation	$Pten^{tm1Hwu/tm1Hwu}$ ($Pten^{flox/flox}$; Tg. Tyr-Cre) conditional inactivation using Tyr-Cre, mice	Ganglionneuromatosis: hypertrophy and hyperplasia of the ENS; fatal intestinal pseudo-obstruction	(Puig et al., 2009)
Other mouse models with hyperganglionosis					
Gdnf	Glial cell line-derived neurotrophic factor	Survival, proliferation, differentiation, and migration	GFAP-Gdnf transgenic mice overexpressing GDNF from GFAP locus circa E18.5	Subtle increase in numbers of submucosal neurons, changes in fiber density, accelerated intestinal transit time	(Wang et al., 2010)
Spry2	Sprouty homolog 2	Negative regulator of GDNF signaling. Alters neonatal development or survival of enteric neurons	$Spry2^{tm1Ayos/tm1Ayos}$ ($Sprouty2^{-/-}$) Targeted gene knockout mice	Enteric neuron hyperplasia, oesophagal achalasia	(Taketomi et al., 2005)
Fgf2	Fibroblast growth factor 2	Unknown	$Fgf2^{tm1Zllr\ /\ tm1Zllr}$ ($Fgf2^{-/-}$) Targeted gene knockout	Hyperplastic enteric ganglia with open architecture of connectives; altered mucosal barrier function and Cl-secretion.	(Hagl et al., 2008; Hagl et al., 2013)
Nog	Noggin	Binds and inactivates members of the TGF-beta superfamily signaling proteins, such as bone morphogenetic protein-4 (BMP4)	NSE-noggin transgenic mice overexpressing noggin under control of the neuron specific enolase promoter	Enteric hyperinnervation. More 5-HT, calretinin, and calbindin. Less γ-aminobutyric acid,TH, DAT, CGRP, and TrkC	(Chalazonitis et al., 2008; Margolis et al., 2011)

2.6.2.2 MOUSE MODELS OF INTESTINAL NEURONAL DYSPLASIA B

T-cell leukemia homeobox 2 (*Tlx2*), also known as *Ncx/Hox11L.1*, is a member of an orphan homeobox-containing transcription factor family. It has a role in the development of enteric nervous system and it is expressed in several tissues derived from the neural crest (Hatano et al., 1997a; Hatano et al., 1997b). Hatano et al. (Hatano et al., 1997a) studied targeted knockout of the *Ncx* gene, *Ncx*$^{-/-}$ mice. All of these mice had hyperganglionic proximal colon and half of these mice also develop mega-ileo-ceco-colon and died between 21–35 days of age. Some of these neuronal cells degenerated and neuronal cell death occurred in later stages. Yamataka et al. (Yamataka et al., 2001) showed that all *Ncx*$^{-/-}$ mice, with and without megacolon, have abnormal innervation in the ileum, cecum and proximal colon. This included hyperganglionosis in both in myenteric and submucosal plexus stained with pan-neuronal marker protein gene product 9.5 (PGP9.5) immunohistochemistry, ectopic cholinergic ganglia in both neuronal plexuses stained with histochemistry, and on nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry ghostlike nitrergic ganglia with blurred edges and branching appearance. In the distal colon, the ganglions were normally distributed. However, one group was unable to reproduce the hyperganglionosis results in *Ncx*$^{-/-}$, even though the mice developed a dysmotility phenotype (Parisi et al., 2003). The abnormal findings were resembling human intestinal neuronal dysplasia B (Toledo de Arruda Lourenção et al., 2016). The megacolon phenotype of *Ncx*$^{-/-}$ is not fully penetrant, and for example, mice in C57BL/6J genetic background are more affected (Parisi et al., 2003). Kato et al. (Kato et al., 2009) studied the proximal colon by immunohistochemistry of membrane bound neural cell adhesion molecule (NCAM) post-translationally modified with poly-sialic acid (PSA). This is a known marker for immature neurons. PSA-NCAM was positive in submucosal and myenteric plexuses of the proximal colon in around half of the *Ncx*$^{-/-}$ mice after P14, when WT mice did not show any staining. It seems that *Ncx/Hox11L.1* is required for maintenance of proper functions of the enteric nervous system or for differentiation (Hatano et al., 1997a; Yamataka et al., 2001; Parisi et al., 2003; Kato et al., 2009). However, the presence of mutations or molecular defects in the *Hox11L.1* coding region in humans with IND-B have not been described (Costa et al., 2000; Fava et al., 2002; Toledo de Arruda Lourenção et al., 2016).

The kinesin superfamily proteins (KIFs) are motor proteins that transport organelles and protein complexes in a microtubule- and ATP-dependent manner. Zhou et al. (Zhou et al., 2009) described targeted gene knockout of *Kif26a* (*Kif26a*$^{-/-}$) in mice leading to death between 7-35 days and megacolon because of GI functional obstruction. *Kif26a*$^{-/-}$ mice showed myenteric hyperganglionosis in the colon, but not in the ileum, with changes being more substantial in the distal colon, where about a 50 % increase in both cholinergic and nitrergic neurons was reported. ENS precursor proliferation was increased at E12.5 in Kif26a$^{-/-}$ mice. Ganglia were less connected by AChE- or NADPH-d-positive fibers in the colon of Kif26a$^{-/-}$ mice and the neurite outgrowth *in vitro* was shorter. Response to cholinergic carbachol stimulus was prolonged in Kif26a$^{-/-}$ mice indicating abnormal coordination of colonic motility. *In vitro*, in a TGW-neuroblastoma cell line, overexpression of *Kif26a* suppressed GDNF/RET mediated ERK and Akt phosphorylation. *In vitro*, RNA interference knock down with targeted microRNA and tissue of primary culture from

Kif26a$^{-/-}$ mice showed upregulation of ERK and Akt cascades. Overexpression and knockout results suggest that KIF26A inhibits GDNF/RET signaling and downstream activation of ERK and Akt cascades, but this has not been shown *in vivo*. Kif26a$^{-/-}$ mice also exhibit differences in the CNS in a form of intense and prolonged nociceptive responses (Wang et al., 2018).

Beside abovementioned mouse models, IND-B type of pathology of giant ganglia in the submucosal plexus has been found in two *Ednrb* knockout heterozygous animal models that do not show gastrointestinal dysmotility symptoms (von Boyen et al., 2002; Holland-Cunz et al., 2003). Homozygous *Ednrb* deficient rats and mice develop HSCR megacolon but these mice do not show IND-B pathology (von Boyen et al., 2002; Holland-Cunz et al., 2003).

Even though in patients IND-B is defined as submucosal hyperganglionosis (Kapur and Reyes-Mugica, 2019), both the in *Ncx*$^{-/-}$ and *Kif26a*$^{-/-}$ mice show hyperplasia present also in the myenteric plexus. Despite this, both of these mouse models are considered to model IND-B. There are small differences in the ENS development between mice and humans, particularly in the development of distal submucosal plexus. In mice, this occurs postnatally and, in humans, early in development (Nagy and Goldstein, 2017). As the diagnostic criteria of IND-B are controversial, it is not clear from human studies if myenteric ganglia were not measured or they were measured but are not different.

2.6.2.3 MOUSE MODELS OF GANGLIONEUROMATOSIS

Okamoto et al. (Okamoto et al., 2019) studied the *RET*(C618F) mutation leading to constitutive activation of RET, which requires a ligand for full activation and causes multiple endocrine neoplasia syndrome (MEN) 2A, leading to medullary thyroid carcinoma and pheochromocytoma. Knock-in mouse line conditionally expressed *RET*(C618F) by *Ret* promoter. *RET*(C618F) could be knocked out by Cre-loxP recombination to start expressing mCherry. Mice expressing *RET*(C618F) displayed mild C cell hyperplasia and around a 20 % increase in the total number of PHOX2B positive myenteric neurons. The submucosal neurons and specific neuronal subtypes where not studied. In MEN2 patients, diffuse intestinal ganglioneuromatosis is solely associated with MEN2B mutations (Gfroerer et al., 2017; Castinetti et al., 2018). This is the opposite to these findings in mice. Also, the level of ganglionic hyperplasia is much more pronounced in MEN2B patients.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) regulates cell size and proliferation by phosphatazing both proteins and phospholipid substrates. PTEN deficiency may alter the MAPK/ERK signaling pathway, and crosstalk to GDNF/GFRa1/RET signaling pathway has been shown (Zbuk and Eng, 2006; Salmena et al., 2008). Because *Pten* knockout is lethal in early embryonic development (Stambolic et al., 1998), a conditional *Pten* knockout approach was used to delete PTEN conditionally from ENS precursors, using transgenic Tyr:Cre line (Delmas et al., 2003; Puig et al., 2009). However, this also affects (or induced Cre recombination in) melanoblasts and a subset of smooth muscle precursors. Homozygous conditional Tyr:Cer *Pten* knockouts died between P13-P20 because of intestinal pseudo-obstruction causing constipation, megacecum and ileal perforations (Puig et al., 2009). These mice showed increased numbers of both neurons and glial cells in myenteric and submucosal plexus. Hyperplasia was occurring after E15.5, when

the gut is fully colonized by ENCCs. Besides hyperplasia, *Pten* cKO mice showed hypertrophy of the neurons after birth. The authors showed that absence of PTEN lead to upregulation of PI3K/PTEN-AKT-S6K signaling pathway and hyperplasia could be prevented with AKT inhibition by antibody treatment. The authors pointed to a link to diffuse ganglionneuromatosis by showing low levels of PTEN in 2/7 studied patient samples and high levels of downstream effector of the PTEN signaling pathway, S6, by immunohistochemistry.

2.6.2.4 MISCELLANEOUS MOUSE MODELS OF HYPERGANGLIONOSIS

Wang et al. used two transgenic approaches and GDNF injections to elevate GDNF levels in mice (Wang et al., 2010). The first transgenic mouse model, Myo-*Gdnf* mice, produced excess GDNF in developing and mature skeletal muscle (Nguyen et al., 1998). *Gdnf* overexpression in muscles did not lead to an ENS phenotype, except for an increase of nitrergic innervation of the esophagus. The expression kinetics for GDNF in the developing intestine were not studied. The, second transgenic mouse model, GFAP-*Gdnf* mice, produced excess GDNF in central nervous system and enteric glia starting around E18 (Zhao et al., 2004). Due to unexplained difficulties in breeding and high mortality as newborns of GFAP-*Gdnf* mice, in a third approach, C57BL/6 mice were subcutaneously injected with recombinant His-tagged GDNF (2 µg/g s.c.) or phosphate buffered saline twice a day for P0-P30. GFAP-*Gdnf* mice and GDNF injected mice had no increase in total number of myenteric neurons. GFAP-Gdnf and GDNF injected mice had small increases in small bowel and colon total neuronal and nitrergic myenteric neuron cell size and in total submucosal neuron density. There was a 30% increase in small bowel and 47% increase in colon NADPH-d neuron density in the myenteric plexus of GFAP-*Gdnf* mice and a 57% increase NADPH-d small bowel myenteric neurons in GDNF injected mice at P30 (Table 2.4). Contraction force was increased in both circular and longitudinal muscle of GFAP-*Gdnf* mice. Also, release of vasoactive intestinal peptide and substance P from the small bowel and colon was increased, despite the transgene increasing the proportion of NADPH-d positive, but there was no effect on ChAT positive neurons (Table 2.4). The ectopic GDNF injections, starting from P1, and transgenic *Gdnf* overexpression, using GFAP promoter starting at E18, resulted in changes in proportions of enteric neurons. These results however, tell little about effects of increased endogenous GDNF in enteric nervous system development and adult function. Besides mouse models, GDNF over expression has been studied with targeted retroviral vector mediated gene transfer *in ovo*, where both decreased and increased GDNF levels caused premature differentiation of enteric neurons (Mwizerwa et al., 2011). The development of the avian enteric nervous system has more similarities than differences, compared to mammals (Heanue et al., 2016). However, this study did not provide data on effects of endogenous GDNF levels on pre,- and postnatal enteric nervous system.

Table 2.4. Key changes in enteric neuronal cell density in GFAP-*Gdnf* and GDNF injected animals summarized from Wang et al. (Wang et al., 2010). Cuprolinic blue staining was used for evaluation of total neuronal density. (=) no change compared to controls, NADPH-d nicotinamide adenine dinucleotide phosphate diaphorase, ChAT choline acetyltransferase.

GFAP-*Gdnf*

small bowel myenteric NADPH-d (+1.3)	>	ChAT (=)
small bowel submucosal total (+1.19)	>	myenteric total (=)
colon myenteric NADPH-d (+1.47)	>	ChAT (=)
colon submucosal total (+1.7)	>	myenteric total (=)

GDNF-injected

small bowel myenteric NADPH-d (+1.57)		ChAT not evaluated
small bowel submucosal total (+2,44)	>	myenteric total (=)
colon myenteric NADPH-d (=)		
colon submucosal total (+3,1)	>	myenteric total (=)

Sprouty and Spred family proteins are evolutionarily conserved inhibitors of tyrosine kinase signaling. Taketomi et al. described targeted gene knockout of Sprouty2 in *Spry2-/-* mice that resulted in the death of half of the knockout mice within 6 weeks from birth (Taketomi et al., 2005). All the mice showed findings resembling esophageal achalasia at different levels accompanied with stronger contraction response to charbachol in lower esophageal sphincter. The phenotype was accompanied with hearing loss. Whole-mount immunostaining with an antibody against the neuronal marker PGP9.5 demonstrated a marked hyperplasticity in the ENS plexus density in the esophagus, ileum and colon. Maximum contraction force in response to cholinergic stimulation was reduced. Anti-GDNF IgG antibody treatment and RET-Fc protein partly rescued the ENS hyperplasia in *Spr2-/-* mice. When colonic tissues from WT and KO mice were incubated in presence and absence of GDNF (50 ng/ml) *in vitro* the authors reported a prolonged phosporylation of Akt and ERK in knockout gut studies with immunohistochemistry and western blotting experiments (Taketomi et al., 2005). In another study, *Spr2-/-* mice were crossed to targeted *Ret*^{Y1062F} knock-in mice, where tyrosine 1062 is replaced with phenylalanine resulting in variating length of aganglionosis or hypoganglionosis and kidney hypoplasia (Jijiwa et al., 2004). This was used to study whether loss of *Spry2* would rescue the ENS or kidney phenotype (Miyamoto et al., 2011). While the effects of loss of *Spry2* for *Ret*^{Y1062F} mice were rather small, it partially rescued kidney hypoplasia and stomach hypoganglionosis, but did not rescue intestinal aganglionosis (Miyamoto et al., 2011).

Fibroblast growth factor (FGF) family members bind heparin and possess broad mitogenic and angiogenic activities. Targeted knockout mice for FGF2 (*Fgf2-/-*) are viable and do not display any obvious neurological deficits (Hagl et al., 2008; Hagl et al., 2013). Whole mount longitudinal muscle myenteric plexus preparations stained with pan-neuronal marker PGP9.5 or cupronilic blue, showed larger enteric neuron size in ganglia with open architecture of connectives in *Fgf2-/-* mice (Hagl et al., 2008). There was no statistically significant difference in contractility in *Fgf2-/-* mice but chlorine secretion measured in Ussing chamber and mucosal barrier function determined with studying bacterial translocation were changed (Hagl et al., 2008). Also, the

proportions of the enteric neuronal subclasses are changed in these mice. There is up to a 40 % reduction of calbindin-positive neurons in wholemount preparations stained for calbindin and calretinin in *Fgf2-/-* mice (Hagl et al., 2013). The precise role of FGF2 in the ENS development is not well established.

Effects of bone morphogenetic protein (BMP) signaling on ENS development were studied in transgenic mice over expressing either the BMP inhibitor, noggin or BMP4 under control of the neuron specific enolase promoter causing transgene over expression in neurons and enteroendocrine cells (Chalazonitis et al., 2008) . Antagonism of the BMP signaling by noggin increased the total numbers of enteric neurons by 40 % and favored the subpopulations derived from precursors that exit the cell cycle early in neurogenesis: serotonin, calretinin and calbindin. The neuronal subpopulations that exit the cell cycle late were reduced. Those included: γ-aminobutyric acid, tyrosine hydroxylase, dopamine transporter, calcitonin gene related peptide, and TrkC positive ENS sub-populations. In contrast, overexpression of BMP4 increased the numbers of TH- and TrkC-expressing neurons. The authors suggested that the data supports the idea that the number of proliferative divisions neuronal precursors undergo before their terminal mitosis, in part, determines the phenotypic expression in the ENS, and that that BMP signaling may regulate enteric neuronal phenotypic diversity by promoting the exit of precursors from the cell cycle. Noggin overexpressing mice had only mild GI functional deficits, mainly irregular rapid GI transit time, stool size, and water content. The same noggin overexpressing mice are more prone to colitis induced with trinitrobenzene sulfonic acid or dextran sulfate sodium (Margolis et al., 2011).

In conclusion animal models of hyperganglionosis are relatively rare. However, GDNF/GFRa1/RET signaling has been linked to most of the existing animal models of hyperganglionosis, either directly or indirectly. Generation of novel animal models would help to understand the development of hyperganglionosis and shed light into the underlying pathogenesis of this rare pathology, which is causing chronic problems to patients who, unlike Hirschsprung's disease patients, often cannot be eased with surgery. It can be speculated that hyperganglionosis may also contribute to more common GI problems, such as constipation or irritable bowel syndrome, since at least in mice even considerable increase in enteric innervation can have only mild effects on GI functions and thus may pass unnoticed. This is even more so in patients, enteric innervation is mostly only studied for diagnosis upon severe chronic obstructions. How variable is the size of the normal ENS innervation density and if and how this links to common GI tract diseases, such as inflammatory bowel disease, is currently unknown. Mouse models of hyperganglionosis may help to shed light into this and provide tools for defining and testing treatment.

3. AIMS OF THE STUDY

The lack of postnatally viable knockout animal models and fundamental issues with transgenic overexpression have hindered the understanding of the role of GDNF/GFRa1/RET signaling in the CNS and ENS development and diseases. The aim of this study was to investigate how GDNF levels influence the brain dopamine system and how the levels of GDNF, and its receptor GFRa1, effect the ENS development and function.

Specific aims of this study were:

I) to analyze the expression site of *Gdnf* mRNA and measure DAT and TH levels and MAPK activity in GDNF hypermorphic mice striata

II) to characterize the phenotype of GFRa1 hypomorphic mice

III) to characterize the role of 3'UTR regulation of GDNF expression in enteric nervous system development and adult function

4. MATERIALS AND METHODS

The methods used by the thesis author in publications I-III are listed in Table 4.1. Detailed descriptions of materials and methods are provided in the original publications. and according supplements. The methods used for additional data included in the thesis are provided in the below supplementary materials and methods.

Table 4.1. Methods used by the author

Method	Used in
In situ hybridization, RNAscope	I
Corridor test*	I
Western blotting	I, II
Microdissection	I, II, III
Genotyping	I, II, III
cDNA synthesis	II, III
Quantitative PCR	II, III
Tissue processing / sectioning	I, II, III
Histochemistry	II, III
Immunohistochemistry	II, III
Microscopy and quantification	I, II, III
GI functional tests	III
ELISA*	III
HPLC	III
BrdU proliferation studies	III
Taqman RT-PCR	III

*) collaboration / non-inpendent

4.1 SUPPLEMENTARY MATERIALS AND METHODS

Crude synaptosomal preparations and membrane protein biotinylation

The dorsal striata were dissected from adult mouse brain (4 months old, male) and the synaptosomal DAT assessment was done as previously described (Kopra et al., 2017). Briefly, the mice were sacrificed by decapitation and the dorsal striata were dissected with a scalpel onto ice from 2 mm slices cut using a mouse brain matrix (Stoelting). The samples were snap-frozen on dry ice and stored at −80°C until assayed.

The striata were cut into approximately equal-size pieces and homogenized with a Teflon pestle in 0.32 M sucrose. One piece was used to measure total DAT protein levels. Crude synaptosomal preparations were prepared as described previously (Hallett et al., 2008). The biotinylated fraction of the synaptosome preparation was then extracted using the Cell Surface Protein Isolation Kit (Pierce) in accordance with the manufacturer's instructions.

Western blotting

The dorsal striatum samples were homogenized on ice in 10 mM HEPES (pH 7.2–7.4), 1 mM EDTA, 0.3 m sucrose, protease inhibitor mixture (Complete Mini-Tabs Protease Inhibitor; Roche) and phosphatase inhibitor (PhosSTOP; Roche). Total protein concentration was measured using the Lowry method (Bio-Rad). A total of 10 µg of protein was separated by SDS-PAGE to detect DAT. The proteins were transferred to a nylon membrane, which was washed 3 times in TBS containing 0.1% Tween 20 (TBS-T) for 15 min and then blocked in 5% (w/v) nonfat milk in TBS-T for 1 h at room temperature (RT). To detect DAT, the membranes were incubated in rat anti-DAT (1:1000, MAB369; Millipore) overnight at 4°C in blocking solution, followed by biotinylated anti-rat antibody (1:500, BA-4000; Vector Laboratories) for 2 h at RT, followed by 2 h at RT with streptavidin-HRP (1:2500, S-911; Invitrogen).

The membranes were stripped for 15 min at 70°C in 50 mM Tris-HCl, pH 7.4, 2% SDS, and 50 mM DTT, and then washed and blocked as described above. The membranes were then incubated in mouse anti-GAPDH antibody (1:10,000 or 1:1000 for total and biotinylated samples, respectively; MAB374; Millipore) for 2 h at 4°C, followed by 2 h at RT in anti-mouse-HRP (1:3000 or 1:1500 for total and biotinylated samples, respectively; P0449; DAKO). The signal was visualized using enhanced chemiluminescence (kit #32106; Pierce), followed by exposure to film. The signal from the gene of interest was normalized to the GAPDH signal using ImageJ software

RNAscope *in situ* hybridization

RNAscope *in situ* hybridization was performed as in (I). Briefly, RNAscope (Wang et al., 2012) probes detecting *Gdnf* (red) and *Pvalb* (green) mRNA were custom made by Advanced Cell Diagnostics and were hybridized to slices from cerebellum and striatum of 3 month old mice, according to manufacturer's recommendations.

Serum urea

Serum urea levels were measured with standard kits (BioAssay Sytems).

Recombinant protein injections

Pregnant dames were given a serial of intravenous injections of 10 µg recombinant fc-GFRa1 fusion protein (R&D systems, catalog number 560-GR/CF) into tail vein from timed pregnancy E10.5 to E15.5. For controlling protein delivery to fetuses E13.5 pregnant NMRI dame was given a single 10 µg injection of fc-GFRa1 and was euthanized one hour later.

Double heterozygous GDNF hypermorphic and GFRa1 hypomorphic animals

GDNF hypermorphic and GFRa1 hypomorphic mouse models were generated by Dr Jaan-Olle Andressoo, as described in the publications I and II respectively. Healthy *Gdnf*$^{WT/hyper}$ and *Gfra1*$^{WT/hypo}$ animals were crossed with each other. Mice were maintained on a 129Ola/ICR/C57BL6 mixed genetic background, housed under a 12h/12h light/dark cycle at 20-22°C, with one mother and litter per cage. Standard chow and water were available *ad libitum*. All animal experiments were approved by the national Animal Experiment Board of Finland.

Immonohistochemistry

Immunohistochemisty of PGP9.5 was performed as described in publications II and III. The number of ganglia was quantified with ImagePro software. For tyrosine hydroxylase and PGP9.5 immunohistochemistry sections were deparaffinized with xylene-alcohol-water series. Antigen retrieval was performed by boiling the samples 10 minutes in fresh 10 mM citrate buffer (pH 6,0 + 0,05 % Tween 20) followed by cooling in the buffer. Quenching of endogenous peroxidase was carried out in 1:53 H2O2 in TBS solution for 30 min at RT. After washing with TBS-T (TBS with 0,1 % Tween 20) blocking was performed in 1,5 % normal goat serum in TBS-T for 30 min at RT. Incubation with primary antibody solution (Rabbit anti ubiquitin C-terminal hydrolase L1 (PGP9.5) 1:250 BML PG9500, Enzo; mouse anti tyrosinehydroxylase, 1:000 MAB318, Millipore) was performed overnight at + 4 °C, incubation with secondary antibody solution (Biotinylated anti-rabbit 1:200 Vector kit; Donkey anti mouse Cy3 Jackson 715-165-150 1:400) was performed for 90 min at RT. After washing in TBS-T, fluorescent paraffin sections were mounted in Immu-mount (Thermo-Scientific). Samples were imaged with Olympus BX-UCB microscope. As negative controls, either the primary or secondary antibodies were omitted.

Gastrointestinal transit time

P7.5 mice were fed a 100 µl bolus of 0.1 % medicinal carbon (Takeda) in 10 % sucrose solution. Mice were euthanized 30 minutes after dye dose and the small intestine transit time was measure as % distance traveled of the total small intestine length.

Experimental design and statistical analysis

All values are presented as mean ± SEM. Statistical significance level was set at $p < 0.05$. Statistical analysis was performed with GraphPad Prism 7.04 software. Appropriate statistical test was used for each dataset: one way ANOVA followed by Tukey's HSD (honestly significant difference) or unpaired two-tailed *t*-test. Animal cohort sizes were selected by the three R principles. Genotypes of quantified samples were blinded from the researcher always when this was technically possible.

Pages 39-66 are under embargo

ACKNOWLEDGEMENTS

This work was carried through at the Institute of Biotechnology and later at the department of Pharmacology, Faculty of Medicine, University of Helsinki during years 2010-2020. However, my path as a graduate student began already year 2007 at the Division of Pharmacology and Toxicology, Faculty of Pharmacy, where I also completed my master studies. In the first few years of my Ph.D. studies I certainly learned that sometimes, but not always, the Murphy's law holds in basic science. After frustrating try to achieve results in studying neurotrophic factors MANF and CDNF *in vitro* and half a year working outside the academia, it was time for me to join the team Jaan-Olle Andressoo, which, at those times, was a part of the Mart Saarma's neurogroup at the Institute of Biotechnology.

I am grateful for my supervisor associate professor Jaan-Olle Andressoo for a second change to start my Ph.D. studies from scratch. These animal models designed by Dr. Andressoo gave me an opportunity to describe and study genetically modified mice with intriguing and obvious phenotypes. I admire professor Andressoo's overwhelming fountain of ideas.

I am thankful to the pre-reviewers of this thesis, assistant professor Meenakshi Rao and docent Annika Meinander, for their constructive and critical comments that helped me to substantially improve the thesis manuscript. I thank Dr. Ulrika Marklund for agreeing to act as my opponent in the public defense of this thesis.

I thank docents Petteri Piepponen and Jari Rossi for their valuable comments in the thesis committee meetings during these years.

I am also thankful to M.Pharm. Heikki Virtanen who joined the lab to carry out his master thesis and help responding the reviewer questions about which Dr. Andressoo kindly phoned me when holding my one day old youngest daughter at Kätilöopisto hospital. I was delighted to get to work with such fast learning and inquisitive person and I thank you for equal contribution to the manuscript (III).

I thank all my co-authors, especially Ph.D. Jaakko Kopra, Ph.D. Anmol Kumar, and my dearest and closest colleague Ph.D. Kärt Mätlik, all former members of Andressoo lab, for work together during these years.

I thank all members or former members of Andressoo lab, Daniel, Giorgio, Soophie, Laoise, Susanna, Ana, Mügen, Jenni, Sakari, Mark, and Rana for scientifically inspiring atmosphere and encouraging but simultaneously relaxed working culture we have achieved together.

I appreciate the work of several technicians who have taught me some of the methods and provided protocols and technician students who have helped to perform some of the experiments.

For funding I thank the Doctoral Program in Biotechnology and Molecular Biology and Finnish Parkinson's foundation.

I thank my former co-workers at the Division of Pharmacology and Toxicology and Raimo K. Tuominen for providing me a chance for my first Ph.D. project attempt and I thank professor Tuominen also as my co-supervisor for this second thesis project and custos for the thesis defense.

I also thank professor Saarma for the facilities and my former co-workers in the Institute of Biotechnology and especially Saarma lab for all the years we spent together at the Viikki campus.

I am truly grateful to my childhood family for the loving atmosphere I was raised in and, especially to my late mother Paula Vilponen, for supporting my education and pushing me forward to strive for life long learning and a Ph.D., which was my goal already at the age of 14.

Last but not least, I thank my dear husband Jaakko Porokuokka for help with the layout of this book and some illustrations during all these years and *par excellence* for your love and devotion to our family. I thank my daughters for the love and laughter that has filled my heart ever since I met you; even though you did not actually much speed up my thesis.

Helsinki, May 2020

REFERENCES

(1981) Harald Hirschsprung: 1830-1916. 24:408.

Abbott RD, Petrovitch H, White LR, Masaki KH, Tanner CM, Curb JD, Grandinetti A, Blanchette PL, Popper JS, Ross GW (2001) Frequency of bowel movements and the future risk of Parkinson's disease. Neurology 57:456-462.

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. Nature reviews Neuroscience 3:383-394.

Al-Rikabi AC, Al-Sohaibani MO, Habeeb A-s, Ahmed A-s, Al Saigh A, Sayah H (2011) Pseudo-obstruction of the Gastric Outlet Caused by Combined Hyperganglionosis and Ganglioneuromatosis in an adult: Case Report and Literature Review. Oman Med J 26:e021-e021.

Albanese V, Lawson VA, Hill AF, Cappai R, Di Guardo G, Staikopoulos V, Thacker M, Furness JB, Chiocchetti R (2008) Evidence for prion protein expression in enteroglial cells of the myenteric plexus of mouse intestine. Autonomic neuroscience : basic & clinical 140:17-23.

Allis CD, Jenuwein T (2016) The molecular hallmarks of epigenetic control. Nature Reviews Genetics 17:487.

Amiel J, Salomon R, Attie T, Pelet A, Trang H, Mokhtari M, Gaultier C, Munnich A, Lyonnet S (1998) Mutations of the RET-GDNF signaling pathway in Ondine's curse. American journal of human genetics 62:715-717.

Amiel J et al. (2008) Hirschsprung disease, associated syndromes and genetics: a review. Journal of medical genetics 45:1-14.

An JJ, Gharami K, Liao G-Y, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B (2008) Distinct Role of Long 3' UTR BDNF mRNA in Spine Morphology and Synaptic Plasticity in Hippocampal Neurons. Cell 134:175-187.

Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A (1996) Germline mutations in glial cell line-derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. Nature genetics 14:341-344.

Annerino DM, Arshad S, Taylor GM, Adler CH, Beach TG, Greene JG (2012) Parkinson's disease is not associated with gastrointestinal myenteric ganglion neuron loss. Acta neuropathologica 124:665-680.

Ano Y, Sakudo A, Nakayama H, Onodera T (2009) Uptake and dynamics of infectious prion protein in the intestine. Protein and peptide letters 16:247-255.

Arthur LL, Chung JJ, Janakirama P, Keefer KM, Kolotilin I, Pavlovic-Djuranovic S, Chalker DL, Grbic V, Green R, Menassa R, True HL, Skeath JB, Djuranovic S (2017) Rapid generation of hypomorphic mutations. Nature Communications 8:14112.

Asai N, Fukuda T, Wu Z, Enomoto A, Pachnis V, Takahashi M, Costantini F (2006) Targeted mutation of serine 697 in the Ret tyrosine kinase causes migration defect of enteric neural crest cells. Development 133:4507-4516.

Ashraf W, Pfeiffer RF, Park F, Lof J, Quigley EM (1997) Constipation in Parkinson's disease: objective assessment and response to psyllium. Movement disorders : official journal of the Movement Disorder Society 12:946-951.

Attie T, Pelet A, Edery P, Eng C, Mulligan LM, Amiel J, Boutrand L, Beldjord C, Nihoul-Fekete C, Munnich A, et al. (1995) Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. Human molecular genetics 4:1381-1386.

Ayanlaja AA, Zhang B, Ji G, Gao Y, Wang J, Kanwore K, Gao D (2018) The reversible effects of glial cell line–derived neurotrophic factor (GDNF) in the human brain. Seminars in Cancer Biology 53:212-222.

Bahuau M, Pelet A, Vidaud D, Lamireau T, LeBail B, Munnich A, Vidaud M, Lyonnet S, Lacombe D (2001) GDNF as a candidate modifier in a type 1 neurofibromatosis (NF1) enteric phenotype. Journal of medical genetics 38:638-643.

Baker DJ (2011) Hypomorphic Mice. In: Transgenic Mouse Methods and Protocols (Hofker MH, van Deursen J, eds), pp 233-244. Totowa, NJ: Humana Press.

Bakheet T, Hitti E, Al-Saif M, Moghrabi WN, Khabar KSA (2018) The AU-rich element landscape across human transcriptome reveals a large proportion in introns and regulation by ELAVL1/HuR. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1861:167-177.

Barbaric I, Miller G, Dear TN (2007) Appearances can be deceiving: phenotypes of knockout mice. Briefings in Functional Genomics 6:91-103.

Barlow A, de Graaff E, Pachnis V (2003) Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron 40:905-916.

Barrenschee M, Böttner M, Hellwig I, Harde J, Egberts JH, Becker T, Wedel TJC, Research T (2013) Site-specific gene expression and localization of growth factor ligand receptors RET, GFRα1 and GFRα2 in human adult colon. 354:371-380.

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136:215-233.

Bassotti G, Villanacci V, Maurer CA, Fisogni S, Di Fabio F, Cadei M, Morelli A, Panagiotis T, Cathomas G, Salerni B (2006) The role of glial cells and apoptosis of enteric neurones in the neuropathology of intractable slow transit constipation. Gut 55:41-46.

Bayliss WM, Starling EH (1899) The movements and innervation of the small intestine. 24:99-143.

Baynash AG, Hosoda K, Giaid A, Richardson JA, Emoto N, Hammer RE, Yanagisawa M (1994) Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell 79:1277-1285.

Bealer JF, Natuzzi ES, Buscher C, Ursell PC, Flake AW, Adzick NS, Harrison MR (1994) Nitric oxide synthase is deficient in the aganglionic colon of patients with Hirschsprung's disease. Pediatrics 93:647-651.

Belkind-Gerson J, Hotta R, Nagy N, Thomas AR, Graham H, Cheng L, Solorzano J, Nguyen D, Kamionek M, Dietrich J, Cherayil BJ, Goldstein AM (2015) Colitis induces enteric neurogenesis through a 5-HT4-dependent mechanism. Inflamm Bowel Dis 21:870-878.

Berkovits BD, Mayr C (2015) Alternative 3' UTRs act as scaffolds to regulate membrane protein localization. Nature 522:363-367.

Berry D, Kuzyk O, Rauch I, Heider S, Schwab C, Hainzl E, Decker T, Müller M, Strobl B, Schleper C, Urich T, Wagner M, Kenner L, Loy A (2015) Intestinal Microbiota Signatures Associated with Inflammation History in Mice Experiencing Recurring Colitis. 6.

Berry D, Schwab C, Milinovich G, Reichert J, Ben Mahfoudh K, Decker T, Engel M, Hai B, Hainzl E, Heider S, Kenner L, Muller M, Rauch I, Strobl B, Wagner M, Schleper C, Urich T, Loy A (2012) Phylotype-level 16S rRNA analysis reveals new bacterial indicators of health state in acute murine colitis. Isme j 6:2091-2106.

Bespalov MM, Sidorova YA, Tumova S, Ahonen-Bishopp A, Magalhaes AC, Kulesskiy E, Paveliev M, Rivera C, Rauvala H, Saarma M (2011) Heparan sulfate proteoglycan syndecan-3 is a novel receptor for GDNF, neurturin, and artemin. The Journal of cell biology 192:153-169.

Besset V, Scott RP, Ibanez CF (2000) Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. The Journal of biological chemistry 275:39159-39166.

Biau S, Jin S, Fan CM (2013) Gastrointestinal defects of the Gas1 mutant involve dysregulated Hedgehog and Ret signaling. Biology open 2:144-155.

Bondurand N, Southard-Smith EM (2016) Mouse models of Hirschsprung disease and other developmental disorders of the enteric nervous system: Old and new players. Developmental biology 417:139-157.

Bornstein JC (2008) Purinergic mechanisms in the control of gastrointestinal motility. Purinergic Signal 4:197-212.

Braak H, Rub U, Gai WP, Del Tredici K (2003) Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. Journal of neural transmission (Vienna, Austria : 1996) 110:517-536.

Braak H, de Vos RA, Bohl J, Del Tredici K (2006a) Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases staged for Parkinson's disease-related brain pathology. Neuroscience letters 396:67-72.

Braak H, Muller CM, Rub U, Ackermann H, Bratzke H, de Vos RA, Del Tredici K (2006b) Pathology associated with sporadic Parkinson's disease--where does it end? Journal of neural transmission Supplementum:89-97.

Branchek TA, Gershon MD (1989) Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. The Journal of comparative neurology 285:262-273.

Brogna A, Ferrara R, Bucceri AM, Lanteri E, Catalano F (1999) Influence of aging on gastrointestinal transit time. An ultrasonographic and radiologic study. Investigative radiology 34:357-359.

Brokhman I, Xu J, Coles BLK, Razavi R, Engert S, Lickert H, Babona-Pilipos R, Morshead CM, Sibley E, Chen C, van der Kooy D (2019) Dual embryonic origin of the mammalian enteric nervous system. Developmental biology 445:256-270.

Bronner ME, Simões-Costa M (2016) The Neural Crest Migrating into the Twenty-First Century. Curr Top Dev Biol 116:115-134.

Bruder E, Meier-Ruge WAJDP (2007) Intestinale neuronale Dysplasie Typ B. 28:137-142.

Brumbaugh J, Di Stefano B, Wang X, Borkent M, Forouzmand E, Clowers KJ, Ji F, Schwarz BA, Kalocsay M, Elledge SJ, Chen Y, Sadreyev RI, Gygi SP, Hu G, Shi Y, Hochedlinger K (2018) Nudt21 Controls Cell Fate by Connecting Alternative Polyadenylation to Chromatin Signaling. Cell 172:106-120.e121.

Butler Tjaden NE, Trainor PA (2013) The developmental etiology and pathogenesis of Hirschsprung disease. Translational research : the journal of laboratory and clinical medicine 162:1-15.

Böttner M, Barrenschee M, Hellwig I, Harde J, Egberts J-H, Becker T, Zorenkov D, Schäfer K-H, Wedel T (2013) The GDNF System Is Altered in Diverticular Disease - Implications for Pathogenesis. PloS one 8:e66290-e66290.

Cantrell VA, Owens SE, Chandler RL, Airey DC, Bradley KM, Smith JR, Southard-Smith EM (2004) Interactions between Sox10 and EdnrB modulate penetrance and severity of aganglionosis in the Sox10Dom mouse model of Hirschsprung disease. Human molecular genetics 13:2289-2301.

Caput D, Beutler B, Hartog K, Thayer R, Brown-Shimer S, Cerami A (1986) Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. Proceedings of the National Academy of Sciences of the United States of America 83:1670-1674.

Carlson KM, Dou S, Chi D, Scavarda N, Toshima K, Jackson CE, Wells SA, Jr., Goodfellow PJ, Donis-Keller H (1994) Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B. Proceedings of the National Academy of Sciences of the United States of America 91:1579-1583.

Carniti C, Belluco S, Riccardi E, Cranston AN, Mondellini P, Ponder BA, Scanziani E, Pierotti MA, Bongarzone I (2006) The Ret(C620R) mutation affects renal and enteric development in a mouse model of Hirschsprung's disease. Am J Pathol 168:1262-1275.

Casini-Raggi V, Kam L, Chong YJ, Fiocchi C, Pizarro TT, Cominelli F (1995) Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. Journal of immunology (Baltimore, Md : 1950) 154:2434-2440.

Castinetti F, Moley J, Mulligan L, Waguespack SG (2018) A comprehensive review on MEN2B. Endocrine-related cancer 25:T29-t39.

Cebrian C, Asai N, D'Agati V, Costantini F (2014) The Number of Fetal Nephron Progenitor Cells Limits Ureteric Branching and Adult Nephron Endowment. Cell reports 7:127-137.

Cersosimo MG, Benarroch EE (2008) Neural control of the gastrointestinal tract: implications for Parkinson disease. Movement disorders : official journal of the Movement Disorder Society 23:1065-1075.

Chalazonitis A, Gershon MD, Greene LA (2012) Cell death and the developing enteric nervous system. Neurochemistry international 61:839-847.

Chalazonitis A, Rothman TP, Chen J, Gershon MD (1998) Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 in vitro and in vivo. Developmental biology 204:385-406.

Chalazonitis A, Pham TD, Li Z, Roman D, Guha U, Gomes W, Kan L, Kessler JA, Gershon MD (2008) Bone morphogenetic protein regulation of enteric neuronal phenotypic diversity: relationship to timing of cell cycle exit. The Journal of comparative neurology 509:474-492.

Challis C, Hori A, Sampson TR, Yoo BB, Challis RC, Hamilton AM, Mazmanian SK, Volpicelli-Daley LA, Gradinaru V (2020) Gut-seeded α-synuclein fibrils promote gut dysfunction and brain pathology specifically in aged mice. Nature neuroscience 23:327-336.

Chen CY, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell 107:451-464.

Cheng Z, Dhall D, Zhao L, Wang HL, Doherty TM, Bresee C, Frykman PK (2010) Murine model of Hirschsprung-associated enterocolitis. I: phenotypic characterization with development of a histopathologic grading system. Journal of pediatric surgery 45:475-482.

Cik M, Masure S, Lesage AS, Van Der Linden I, Van Gompel P, Pangalos MN, Gordon RD, Leysen JE (2000) Binding of GDNF and neurturin to human GDNF family receptor alpha 1 and 2. Influence of cRET and cooperative interactions. The Journal of biological chemistry 275:27505-27512.

Clairembault T, Kamphuis W, Leclair-Visonneau L, Rolli-Derkinderen M, Coron E, Neunlist M, Hol EM, Derkinderen P (2014) Enteric GFAP expression and phosphorylation in Parkinson's disease. Journal of neurochemistry 130:805-815.

Coerdt W, Michel JS, Rippin G, Kletzki S, Gerein V, Muntefering H, Arnemann J (2004) Quantitative morphometric analysis of the submucous plexus in age-related control groups. Virchows Archiv : an international journal of pathology 444:239-246.

Cohen S, Levi-Montalcini R (1957) Purification and properties of a nerve growth-promoting factor isolated from mouse sarcoma 180. Cancer research 17:15-20.

Collins J, Borojevic R, Verdu EF, Huizinga JD, Ratcliffe EM (2014) Intestinal microbiota influence the early postnatal development of the enteric nervous system. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 26:98-107.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science (New York, NY) 339:819-823.

Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ (1996) Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75:949-967.

Costa M, Fava M, Seri M, Cusano R, Sancandi M, Forabosco P, Lerone M, Martucciello G, Romeo G, Ceccherini I (2000) Evaluation of the HOX11L1 gene as a candidate for congenital disorders of intestinal innervation. Journal of medical genetics 37:E9.

Costantini F, Shakya R (2006) GDNF/Ret signaling and the development of the kidney. BioEssays : news and reviews in molecular, cellular and developmental biology 28:117-127.

de Graaff E, Srinivas S, Kilkenny C, D'Agati V, Mankoo BS, Costantini F, Pachnis V (2001) Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. Genes & development 15:2433-2444.

De Vadder F, Grasset E, Manneras Holm L, Karsenty G, Macpherson AJ, Olofsson LE, Backhed F (2018) Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. Proceedings of the National Academy of Sciences of the United States of America 115:6458-6463.

de Vicente JC, Cabo R, Ciriaco E, Laurà R, Naves FJ, Silos-Santiago I, Vega JA (2002) Impaired dental cytodifferentiation in Glial cell-line derived growth factor (GDNF) deficient mice. Annals of Anatomy - Anatomischer Anzeiger 184:85-92.

Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes & development 25:1010-1022.

Dekkers MPJ, Nikoletopoulou V, Barde Y-A (2013) Cell biology in neuroscience: Death of developing neurons: new insights and implications for connectivity. The Journal of cell biology 203:385-393.

Del Rey NL-G, Quiroga-Varela A, Garbayo E, Carballo-Carbajal I, Fernández-Santiago R, Monje MHG, Trigo-Damas I, Blanco-Prieto MJ, Blesa J (2018) Advances in Parkinson's Disease: 200 Years Later. 12.

Delmas V, Martinozzi S, Bourgeois Y, Holzenberger M, Larue L (2003) Cre-mediated recombination in the skin melanocyte lineage. Genesis (New York, NY : 2000) 36:73-80.

Demehri FR, Halaweish IF, Coran AG, Teitelbaum DH (2013) Hirschsprung-associated enterocolitis: pathogenesis, treatment and prevention. Pediatric surgery international 29:873-881.

Derti A, Garrett-Engele P, Macisaac KD, Stevens RC, Sriram S, Chen R, Rohl CA, Johnson JM, Babak T (2012) A quantitative atlas of polyadenylation in five mammals. Genome Res 22:1173-1183.

Di Nardo G, Stanghellini V, Cucchiara S, Barbara G, Pasquinelli G, Santini D, Felicani C, Grazi G, Pinna AD, Cogliandro R, Cremon C, Gori A, Corinaldesi R, Sanders KM, De Giorgio R (2006) Enteric neuropathology of congenital intestinal obstruction: A case report. World J Gastroenterol 12:5229-5233.

Di Rienzi SC, Sharon I, Wrighton KC, Koren O, Hug LA, Thomas BC, Goodrich JK, Bell JT, Spector TD, Banfield JF, Ley RE (2013) The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. Elife 2:e01102-e01102.

Doray B, Salomon R, Amiel J, Pelet A, Touraine R, Billaud M, Attie T, Bachy B, Munnich A, Lyonnet S (1998) Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. Human molecular genetics 7:1449-1452.

Doyle A, McGarry MP, Lee NA, Lee JJ (2012) The construction of transgenic and gene knockout/knockin mouse models of human disease. Transgenic research 21:327-349.

Druckenbrod NR, Epstein ML (2005) The pattern of neural crest advance in the cecum and colon. Developmental biology 287:125-133.

Durbec PL, Larsson-Blomberg LB, Schuchardt A, Costantini F, Pachnis V (1996) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development 122:349-358.

Eketjall S, Ibanez CF (2002) Functional characterization of mutations in the GDNF gene of patients with Hirschsprung disease. Human molecular genetics 11:325-329.

El-Brolosy MA, Stainier DYR (2017) Genetic compensation: A phenomenon in search of mechanisms. PLoS genetics 13:e1006780-e1006780.

El Aidy S, Derrien M, Aardema R, Hooiveld G, Richards SE, Dane A, Dekker J, Vreeken R, Levenez F, Dore J, Zoetendal EG, van Baarlen P, Kleerebezem M (2014) Transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonisation of germ-free mice. Beneficial microbes 5:67-77.

Eng C (1996) The RET Proto-Oncogene in Multiple Endocrine Neoplasia Type 2 and Hirschsprung's Disease. 335:943-951.

Enomoto Hideki AT, Jackman Alana, Heuckeroth Robert O., Snider William D., Johnson, Eugene M. Jr., Milbrandt Jeffrey (1998) GFRa1-Deficient Mice Have Deficits in the Enteric Nervous System and Kidneys. Neuron 21:317–324.

Fava M, Borghini S, Cinti R, Cusano R, Seri M, Lerone M, De Giorgio R, Stanghellini V, Martucciello G, Ravazzolo R, Ceccherini I (2002) HOX11L1: a promoter study to evaluate possible expression defects in intestinal motility disorders. International journal of molecular medicine 10:101-106.

Feichter S, Meier-Ruge WA, Bruder E (2009) The histopathology of gastrointestinal motility disorders in children. Seminars in pediatric surgery 18:206-211.

Fernandez RM, Sanchez-Mejias A, Ruiz-Ferrer MM, Lopez-Alonso M, Antinolo G, Borrego S (2009) Is the RET proto-oncogene involved in the pathogenesis of intestinal neuronal dysplasia type B? Molecular medicine reports 2:265-270.

Fitze G, Schierz M, Kuhlisch E, Schreiber M, Ziegler A, Roesner D, Schackert HK (2003) Novel intronic polymorphisms in the RET proto-oncogene and their association with Hirschsprung disease. Human mutation 22:177.

Focke PJ, Schiltz CA, Jones SE, Watters JJ, Epstein ML (2001) Enteric neuroblasts require the phosphatidylinositol 3-kinase pathway for GDNF-stimulated proliferation. Journal of neurobiology 47:306-317.

Freimer JW, Hu TJ, Blelloch R (2018) Decoupling the impact of microRNAs on translational repression versus RNA degradation in embryonic stem cells. Elife 7:e38014.

Frett B, Carlomagno F, Moccia ML, Brescia A, Federico G, De Falco V, Admire B, Chen Z, Qi W, Santoro M, Li HY (2015) Fragment-Based Discovery of a Dual pan-RET/VEGFR2 Kinase Inhibitor Optimized for Single-Agent Polypharmacology. Angewandte Chemie (International ed in English) 54:8717-8721.

Friedman RC, Farh KK-H, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19:92-105.

Frykman PK, Short SS (2012) Hirschsprung-associated enterocolitis: prevention and therapy. Seminars in pediatric surgery 21:328-335.

Fujimoto T (1988) Natural history and pathophysiology of enterocolitis in the piebald lethal mouse model of Hirschsprung's disease. Journal of pediatric surgery 23:237-242.

Furness JB (2012) The enteric nervous system and neurogastroenterology. Nature reviews Gastroenterology & hepatology 9:286-294.

Furness JB, Stebbing MJ (2018) The first brain: Species comparisons and evolutionary implications for the enteric and central nervous systems. 30:e13234.

Furness JB, Callaghan BP, Rivera LR, Cho HJ (2014) The enteric nervous system and gastrointestinal innervation: integrated local and central control. Advances in experimental medicine and biology 817:39-71.

Gabella G, Trigg P (1984) Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbits and sheep. Journal of Neurocytology 13:49-71.

Gaide O, Schneider P (2003) Permanent correction of an inherited ectodermal dysplasia with recombinant EDA. Nature medicine 9:614-618.

Gans C, Northcutt RG (1983) Neural crest and the origin of vertebrates: a new head. Science (New York, NY) 220:268-273.

Garneau NL, Wilusz J, Wilusz CJ (2007) The highways and byways of mRNA decay. Nat Rev Mol Cell Biol 8:113-126.

Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, Simmerman L, Russell D, Martin D, Lapchak PA, Collins F, Hoffer BJ, Gerhardt GA (1996) Functional recovery in parkinsonian monkeys treated with GDNF. Nature 380:252-255.

Gath R, Goessling A, Keller KM, Koletzko S, Coerdt W, Muntefering H, Wirth S, Hofstra RM, Mulligan L, Eng C, von Deimling A (2001) Analysis of the RET, GDNF, EDN3, and EDNRB genes in patients with intestinal neuronal dysplasia and Hirschsprung disease. Gut 48:671-675.

Gershon MD (1999) The enteric nervous system: a second brain. Hospital practice (1995) 34:31-32, 35-38, 41-32 passim.

Gershon MD, Ratcliffe EM (2004) Developmental biology of the enteric nervous system: pathogenesis of Hirschsprung's disease and other congenital dysmotilities. Seminars in pediatric surgery 13:224-235.

Gevers D et al. (2014) The treatment-naive microbiome in new-onset Crohn's disease. Cell host & microbe 15:382-392.

Gfroerer S, Theilen TM, Fiegel H, Harter PN, Mittelbronn M, Rolle U (2017) Identification of intestinal ganglioneuromatosis leads to early diagnosis of MEN2B: role of rectal biopsy. Journal of pediatric surgery 52:1161-1165.

Gianino S, Grider JR, Cresswell J, Enomoto H, Heuckeroth RO (2003) GDNF availability determines enteric neuron number by controlling precursor proliferation. Development 130:2187-2198.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nature medicine 9:589-595.

Golden JP, Baloh RH, Kotzbauer PT, Lampe PA, Osborne PA, Milbrandt J, Johnson EM, Jr. (1998) Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. The Journal of comparative neurology 398:139-150.

Goldstein A, Hofstra R, Burns A (2013) Building a brain in the gut: development of the enteric nervous system. 83:307-316.

Gosain A, Brinkman AS (2015) Hirschsprung's associated enterocolitis. Curr Opin Pediatr 27:364-369.

Grimm SA et al. (2019) DNA methylation in mice is influenced by genetics as well as sex and life experience. Nature Communications 10:305.

Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Molecular cell 27:91-105.

Griseri P, Lantieri F, Puppo F, Bachetti T, Di Duca M, Ravazzolo R, Ceccherini I (2007) A common variant located in the 3'UTR of the RET gene is associated with protection from Hirschsprung disease. Human mutation 28:168-176.

Gruber AJ, Schmidt R, Gruber AR, Martin G, Ghosh S, Belmadani M, Keller W, Zavolan M (2016) A comprehensive analysis of 3' end sequencing data sets reveals novel polyadenylation signals and the repressive role of heterogeneous ribonucleoprotein C on cleavage and polyadenylation. Genome Res 26:1145-1159.

Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science (New York, NY) 265:103-106.

Guan C, Ye C, Yang X, Gao J (2010) A review of current large-scale mouse knockout efforts. Genesis (New York, NY : 2000) 48:73-85.

Guay C, Roggli E, Nesca V, Jacovetti C, Regazzi R (2011) Diabetes mellitus, a microRNA-related disease? Translational research : the journal of laboratory and clinical medicine 157:253-264.

Gujral TS, Mulligan LM (2006) Molecular Implications of RET Mutations for Pheochromocytoma Risk in Multiple Endocrine Neoplasia 2. 1073:234-240.

Gurumurthy CB, Lloyd KCK (2019) Generating mouse models for biomedical research: technological advances. 12:dmm029462.

Hagl CI, Wink E, Scherf S, Heumuller-Klug S, Hausott B, Schafer KH (2013) FGF2 deficit during development leads to specific neuronal cell loss in the enteric nervous system. Histochemistry and cell biology 139:47-57.

Hagl CI, Klotz M, Wink E, Kranzle K, Holland-Cunz S, Gretz N, Diener M, Schafer KH (2008) Temporal and regional morphological differences as a consequence of FGF-2 deficiency are mirrored in the myenteric proteome. Pediatric surgery international 24:49-60.

Hall B, Limaye A, Kulkarni AB (2009) Overview: generation of gene knockout mice. Curr Protoc Cell Biol Chapter 19:Unit-19.12.17.

Hallett PJ, Collins TL, Standaert DG, Dunah AW (2008) Biochemical Fractionation of Brain Tissue for Studies of Receptor Distribution and Trafficking. 42:1.16.11-11.16.16.

Hamburger V, Levi-Montalcini R (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. The Journal of experimental zoology 111:457-501.

Hancock DB, Martin ER, Mayhew GM, Stajich JM, Jewett R, Stacy MA, Scott BL, Vance JM, Scott WK (2008) Pesticide exposure and risk of Parkinson's disease: a family-based case-control study. BMC Neurol 8:6-6.

Hansford JR, Mulligan LM (2000) Multiple endocrine neoplasia type 2 and RET: from neoplasia to neurogenesis. Journal of medical genetics 37:817-827.

Hashino E, Shero M, Junghans D, Rohrer H, Milbrandt J, Johnson EM (2001) GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. 128:3773-3782.

Hatano M, Aoki T, Dezawa M, Yusa S, Iitsuka Y, Koseki H, Taniguchi M, Tokuhisa T (1997a) A novel pathogenesis of megacolon in Ncx/Hox11L.1 deficient mice. The Journal of clinical investigation 100:795-801.

Hatano M, Iitsuka Y, Yamamoto H, Dezawa M, Yusa S, Kohno Y, Tokuhisa TJA, Embryology (1997b) Ncx, a Hox11 related gene, is expressed in a variety of tissues derived from neural crest cells. 195:419-425.

Heanue TA, Pachnis V (2007) Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies. Nature Reviews Neuroscience 8:466-479.

Heanue TA, Shepherd IT, Burns AJ (2016) Enteric nervous system development in avian and zebrafish models. Developmental biology 417:129-138.

Hearn CJ, Murphy M, Newgreen D (1998) GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Developmental biology 197:93-105.

Hellmich HL, Kos L, Cho ES, Mahon KA, Zimmer A (1996) Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. Mechanisms of development 54:95-105.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vandlen RA, Simpson LCctSL, Koliatsos VE, Rosenthal A, et al. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science (New York, NY) 266:1062-1064.

Heuckeroth RO (2003) Finding your way to the end: a tale of GDNF and endothelin-3. Neuron 40:871-873.

Heuckeroth RO (2018) Hirschsprung disease — integrating basic science and clinical medicine to improve outcomes. Nature Reviews Gastroenterology &Amp; Hepatology 15:152.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EM, Jr., Milbrandt J (1999) Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. Neuron 22:253-263.

Hidalgo-Figueroa M, Bonilla S, Gutierrez F, Pascual A, Lopez-Barneo J (2012) GDNF is predominantly expressed in the PV+ neostriatal interneuronal ensemble in normal mouse and after injury of the nigrostriatal pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:864-872.

Higgins PD, Johanson JF (2004) Epidemiology of constipation in North America: a systematic review. The American journal of gastroenterology 99:750-759.

Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, Lin LF, Gerhardt GA (1994) Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. Neuroscience letters 182:107-111.

Hoffmann C, Hill DA, Minkah N, Kirn T, Troy A, Artis D, Bushman F (2009) Community-wide response of the gut microbiota to enteropathogenic Citrobacter rodentium infection revealed by deep sequencing. Infection and immunity 77:4668-4678.

Hofmann AD, Duess JW, Puri PJPSI (2014) Congenital anomalies of the kidney and urinary tract (CAKUT) associated with Hirschsprung's disease: a systematic review. 30:757-761.

Hofstra RM, Valdenaire O, Arch E, Osinga J, Kroes H, Loffler BM, Hamosh A, Meijers C, Buys CH (1999) A loss-of-function mutation in the endothelin-converting enzyme 1 (ECE-1) associated with Hirschsprung disease, cardiac defects, and autonomic dysfunction. American journal of human genetics 64:304-308.

Hofstra RM, Osinga J, Tan-Sindhunata G, Wu Y, Kamsteeg EJ, Stulp RP, van Ravenswaaij-Arts C, Majoor-Krakauer D, Angrist M, Chakravarti A, Meijers C, Buys CH (1996) A homozygous mutation in the endothelin-3 gene associated with a combined Waardenburg type 2 and Hirschsprung phenotype (Shah-Waardenburg syndrome). Nature genetics 12:445-447.

Hofstra RMW, Wu Y, Stulp RP, Elfferich P, Osinga J, Maas SM, Siderius L, Brooks AS, vd Ende JJ, Heydendael VMR, Severijnen RSVM, Bax KMA, Meijers C, Buys CHCM (2000) RET and GDNF gene scanning in Hirschsprung patients using two dual denaturing gel systems. 15:418-429.

Holland-Cunz S, Krammer HJ, Suss A, Tafazzoli K, Wedel T (2003) Molecular genetics of colorectal motility disorders. European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery [et al] = Zeitschrift fur Kinderchirurgie 13:146-151.

Hooper LV, Stappenbeck TS, Hong CV, Gordon JI (2003) Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nature Immunology 4:269-273.

Hoque M, Ji Z, Zheng D, Luo W, Li W, You B, Park JY, Yehia G, Tian B (2013) Analysis of alternative cleavage and polyadenylation by 3' region extraction and deep sequencing. Nature methods 10:133-139.

Hosoda K, Hammer RE, Richardson JA, Baynash AG, Cheung JC, Giaid A, Yanagisawa M (1994) Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. Cell 79:1267-1276.

Ikeda K, Mason PJ, Bessler M (2011) 3'UTR-truncated Hmga2 cDNA causes MPN-like hematopoiesis by conferring a clonal growth advantage at the level of HSC in mice. 117:5860-5869.

Imhann F, Vich Vila A, Bonder MJ, Fu J, Gevers D, Visschedijk MC, Spekhorst LM, Alberts R, Franke L, van Dullemen HM, Ter Steege RWF, Huttenhower C, Dijkstra G, Xavier RJ, Festen EAM, Wijmenga C, Zhernakova A, Weersma RK (2018) Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. Gut 67:108-119.

Ivanchuk SM, Myers SM, Eng C, Mulligan LM (1996) De novo mutation of GDNF, ligand for the RET/GDNFR-alpha receptor complex, in Hirschsprung disease. Human molecular genetics 5:2023-2026.

Jaenisch R, Mintz B (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proceedings of the National Academy of Sciences of the United States of America 71:1250-1254.

Jain S, Naughton CK, Yang M, Strickland A, Vij K, Encinas M, Golden J, Gupta A, Heuckeroth R, Johnson EM, Jr., Milbrandt J (2004) Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. Development 131:5503-5513.

Jalkanen AL, Coleman SJ, Wilusz J (2014) Determinants and implications of mRNA poly(A) tail size--does this protein make my tail look big? Seminars in cell & developmental biology 34:24-32.

Jasin M, Rothstein R (2013) Repair of strand breaks by homologous recombination. Cold Spring Harbor perspectives in biology 5:a012740.

Jiang Q, Ho Y-Y, Hao L, Nichols Berrios C, Chakravarti A (2011) Copy number variants in candidate genes are genetic modifiers of Hirschsprung disease. PloS one 6:e21219-e21219.

Jijiwa M, Fukuda T, Kawai K, Nakamura A, Kurokawa K, Murakumo Y, Ichihara M, Takahashi M (2004) A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. Mol Cell Biol 24:8026-8036.

Jin S, Martinelli DC, Zheng X, Tessier-Lavigne M, Fan C-M (2015) Gas1 is a receptor for sonic hedgehog to repel enteric axons. 112:E73-E80.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 85:1113-1124.

Johansson ME, Hansson GC (2013) Mucus and the goblet cell. Digestive diseases (Basel, Switzerland) 31:305-309.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human MicroRNA targets. PLoS biology 2:e363.

Jones PL, Veenstra GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nature genetics 19:187-191.

Jost WH (1997) Gastrointestinal motility problems in patients with Parkinson's disease. Effects of antiparkinsonian treatment and guidelines for management. Drugs & aging 10:249-258.

Kakoki M, Tsai Y-S, Kim H-S, Hatada S, Ciavatta DJ, Takahashi N, Arnold LW, Maeda N, Smithies O (2004) Altering the Expression in Mice of Genes by Modifying Their 3' Regions. Developmental Cell 6:597-606.

Kallijarvi J, Stratoulias V, Virtanen K, Hietakangas V, Heino TI, Saarma M (2012) Characterization of Drosophila GDNF receptor-like and evidence for its evolutionarily conserved interaction with neural cell adhesion molecule (NCAM)/FasII. PloS one 7:e51997.

Kalueff AV, Ren-Patterson RF, Murphy DL (2007) The developing use of heterozygous mutant mouse models in brain monoamine transporter research. Trends in pharmacological sciences 28:122-127.

Kapur RP (2000) Developmental disorders of the enteric nervous system. 47:iv81-iv83.

Kapur RP, Reyes-Mugica M (2019) Intestinal Neuronal Dysplasia Type B: An Updated Review of a Problematic Diagnosis. 143:235-243.

Kato Y, Miyahara K, Hatano M, Hasegawa Y, Seki T, Frykman PK, Kusafuka J, Lane GJ, Yamataka AJPSI (2009) Immature enteric neurons in Ncx/Hox11L.1 deficient intestinal neuronal dysplasia model mice. 25:961.

Kenny SE, Tam PK, Garcia-Barcelo M (2010) Hirschsprung's disease. Seminars in pediatric surgery 19:194-200.

Kholodilov N, Yarygina O, Oo TF, Zhang H, Sulzer D, Dauer W, Burke RE (2004) Regulation of the development of mesencephalic dopaminergic systems by the selective expression of glial cell line-derived neurotrophic factor in their targets. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:3136-3146.

Kim M, Kim DJ (2018) GFRA1: A Novel Molecular Target for the Prevention of Osteosarcoma Chemoresistance. Int J Mol Sci 19:1078.

Kim S, Kwon SH, Kam TI, Panicker N, Karuppagounder SS, Lee S, Lee JH, Kim WR, Kook M, Foss CA, Shen C, Lee H, Kulkarni S, Pasricha PJ, Lee G, Pomper MG, Dawson VL, Dawson TM, Ko HS (2019) Transneuronal Propagation of Pathologic alpha-Synuclein from the Gut to the Brain Models Parkinson's Disease. Neuron 103:627-641.e627.

Klingelhoefer L, Reichmann H (2015) Pathogenesis of Parkinson disease--the gut-brain axis and environmental factors. Nature reviews Neurology 11:625-636.

Kobayashi H, Hirakawa H, Surana R, O'Briain DS, Puri P (1995) Intestinal neuronal dysplasia is a possible cause of persistent bowel symptoms after pull-through operation for Hirschsprung's disease. Journal of pediatric surgery 30:253-259.

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G (1999) Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. Immunity 10:387-398.

Kopra J, Vilenius C, Grealish S, Harma MA, Varendi K, Lindholm J, Castren E, Voikar V, Bjorklund A, Piepponen TP, Saarma M, Andressoo JO (2015) GDNF is not required for catecholaminergic neuron survival in vivo. Nature neuroscience 18:319-322.

Kopra JJ, Panhelainen A, af Bjerkén S, Porokuokka LL, Varendi K, Olfat S, Montonen H, Piepponen TP, Saarma M, Andressoo J-O (2017) Dampened Amphetamine-Stimulated Behavior and Altered Dopamine Transporter Function in the Absence of Brain GDNF. 37:1581-1590.

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Cochran EJ, Mufson EJ, Penn R, Goetz CG, Comella CD (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Annals of neurology 46:419-424.

Kulkarni S et al. (2017) Adult enteric nervous system in health is maintained by a dynamic balance between neuronal apoptosis and neurogenesis. 114:E3709-E3718.

Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America 89:6232-6236.

Lambert SA, Jolma A, Campitelli LF, Das PK, Yin Y, Albu M, Chen X, Taipale J, Hughes TR, Weirauch MT (2018) The Human Transcription Factors. Cell 172:650-665.

Lander ES (2016) The Heroes of CRISPR. Cell 164:18-28.

Lang AE et al. (2006) Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Annals of neurology 59:459-466.

Laranjeira C, Sandgren K, Kessaris N, Richardson W, Potocnik A, Vanden Berghe P, Pachnis V (2011) Glial cells in the mouse enteric nervous system can undergo neurogenesis in response to injury. The Journal of Clinical Investigation 121:3412-3424.

Lawson VA, Furness JB, Klemm HM, Pontell L, Chan E, Hill AF, Chiocchetti R (2010) The brain to gut pathway: a possible route of prion transmission. Gut 59:1643-1651.

Le Berre-Scoul C, Chevalier J, Oleynikova E, Cossais F, Talon S, Neunlist M, Boudin H (2017) A novel enteric neuron-glia coculture system reveals the role of glia in neuronal development. J Physiol 595:583-598.

Le Douarin NM, Teillet MA (1973) The migration of neural crest cells to the wall of the digestive tract in avian embryo. Journal of embryology and experimental morphology 30:31-48.

Le Douarin NM, Teillet MA (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. Developmental biology 41:162-184.

Lebouvier T, Neunlist M, Bruley des Varannes S, Coron E, Drouard A, N'Guyen JM, Chaumette T, Tasselli M, Paillusson S, Flamand M, Galmiche JP, Damier P, Derkinderen P (2010) Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms. PloS one 5:e12728.

Ledda F, Paratcha G, Sandoval-Guzman T, Ibanez CF (2007) GDNF and GFRalpha1 promote formation of neuronal synapses by ligand-induced cell adhesion. Nature neuroscience 10:293-300.

Lee I, Ajay SS, Yook JI, Kim HS, Hong SH, Kim NH, Dhanasekaran SM, Chinnaiyan AM, Athey BD (2009) New class of microRNA targets containing simultaneous 5'-UTR and 3'-UTR interaction sites. Genome Res 19:1175-1183.

Lee TI, Young RA (2013) Transcriptional regulation and its misregulation in disease. Cell 152:1237-1251.

Leenders E, Sieber WK (1970) Congenital megacolon observation by Frederick Ruysch--1691. Journal of pediatric surgery 5:1-3.

Lelli KM, Slattery M, Mann RS (2012) Disentangling the Many Layers of Eukaryotic Transcriptional Regulation. 46:43-68.

Leva GD, Garofalo M, Croce CM (2014) MicroRNAs in Cancer. 9:287-314.

Levi-Montalcini R, Cohen S (1956) IN VITRO AND IN VIVO EFFECTS OF A NERVE GROWTH-STIMULATING AGENT ISOLATED FROM SNAKE VENOM. Proceedings of the National Academy of Sciences of the United States of America 42:695-699.

Levine M, Tjian R (2003) Transcription regulation and animal diversity. Nature 424:147-151.

Levine M, Davidson EH (2005) Gene regulatory networks for development. Proceedings of the National Academy of Sciences of the United States of America 102:4936-4942.

Li H, Jakobson M, Ola R, Gui Y, Kumar A, Sipilä P, Sariola H, Kuure S, Andressoo J-O (2019a) Development of the urogenital system is regulated via the 3'UTR of GDNF. Scientific reports 9:5302.

Li Z, Hao MM, Van den Haute C, Baekelandt V, Boesmans W, Vanden Berghe P (2019b) Regional complexity in enteric neuron wiring reflects diversity of motility patterns in the mouse large intestine. Elife 8:e42914.

Lianoglou S, Garg V, Yang JL, Leslie CS, Mayr C (2013) Ubiquitously transcribed genes use alternative polyadenylation to achieve tissue-specific expression. Genes & development 27:2380-2396.

Liao G-Y, An JJ, Gharami K, Waterhouse EG, Vanevski F, Jones KR, Xu B (2012) Dendritically targeted Bdnf mRNA is essential for energy balance and response to leptin. Nature medicine 18:564-571.

Lin L, Doherty D, Lile J, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. 260:1130-1132.

Lindahl M, Saarma M, Lindholm P (2017) Unconventional neurotrophic factors CDNF and MANF: Structure, physiological functions and therapeutic potential. Neurobiology of disease 97:90-102.

Liu GX, Yang YX, Yan J, Zhang T, Zou YP, Huang XL, Gan HT (2014) Glial-derived neurotrophic factor reduces inflammation and improves delayed colonic transit in rat models of dextran sulfate sodium-induced colitis. International immunopharmacology 19:145-152.

Loy A, Pfann C, Steinberger M, Hanson B, Herp S, Brugiroux S, Gomes Neto JC, Boekschoten MV, Schwab C, Urich T, Ramer-Tait AE, Rattei T, Stecher B, Berry D (2017) Lifestyle and Horizontal Gene Transfer-Mediated Evolution of Mucispirillum schaedleri, a Core Member of the Murine Gut Microbiota. mSystems 2.

Lui VC, Samy ET, Sham MH, Mulligan LM, Tam PK (2002) Glial cell line-derived neurotrophic factor family receptors are abnormally expressed in aganglionic bowel of a subpopulation of patients with Hirschsprung's disease. Laboratory investigation; a journal of technical methods and pathology 82:703-712.

Luo R, Bai C, Yang L, Zheng Z, Su G, Gao G, Wei Z, Zuo Y, Li G (2018) DNA methylation subpatterns at distinct regulatory regions in human early embryos. Open Biology 8:180131.

Madsen JL, Graff J (2004) Effects of ageing on gastrointestinal motor function. Age and ageing 33:154-159.

Majid S, Dar AA, Saini S, Yamamura S, Hirata H, Tanaka Y, Deng G, Dahiya R (2010) MicroRNA-205-directed transcriptional activation of tumor suppressor genes in prostate cancer. Cancer 116:5637-5649.

Margolis KG, Stevanovic K, Karamooz N, Li ZS, Ahuja A, D'Autréaux F, Saurman V, Chalazonitis A, Gershon MD (2011) Enteric Neuronal Density Contributes to the Severity of Intestinal Inflammation. Gastroenterology 141:588-598.e582.

Martin GR, Alvarez AL, Bashashati M, Keenan CM, Jirik FR, Sharkey KA (2012) Endogenous cellular prion protein regulates contractility of the mouse ileum. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 24:e412-424.

Matlik K, Voikar V, Vilenius C, Kulesskaya N, Andressoo JO (2018) Two-fold elevation of endogenous GDNF levels in mice improves motor coordination without causing side-effects. Scientific reports 8:11861.

Matsui M, Chu Y, Zhang H, Gagnon KT, Shaikh S, Kuchimanchi S, Manoharan M, Corey DR, Janowski BA (2013) Promoter RNA links transcriptional regulation of inflammatory pathway genes. Nucleic acids research 41:10086-10109.

Mattar AF, Coran AG, Teitelbaum DH (2003) MUC-2 mucin production in Hirschsprung's disease: possible association with enterocolitis development. Journal of pediatric surgery 38:417-421; discussion 417-421.

Mayr C (2016) Evolution and Biological Roles of Alternative 3'UTRs. Trends Cell Biol 26:227-237.

Mayr C (2017) Regulation by 3'-Untranslated Regions. 51:171-194.

Mayr C (2018) What Are 3' UTRs Doing? Cold Spring Harbor perspectives in biology.

Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122:107-118.

McCallion AS, Stames E, Conlon RA, Chakravarti A (2003) Phenotype variation in two-locus mouse models of Hirschsprung disease: tissue-specific interaction between Ret and Ednrb. Proceedings of the National Academy of Sciences of the United States of America 100:1826-1831.

McGuire MH, Herbrich SM, Dasari SK, Wu SY, Wang Y, Rupaimoole R, Lopez-Berestein G, Baggerly KA, Sood AK (2019) Pan-cancer genomic analysis links 3'UTR DNA methylation with increased gene expression in T cells. EBioMedicine 43:127-137.

McKeown SJ, Wallace AS, Anderson RB (2013) Expression and function of cell adhesion molecules during neural crest migration. Developmental biology 373:244-257.

Meier-Ruge W (1971) [Casuistic of colon disorder with symptoms of Hirschsprung's disease (author's transl)]. Verhandlungen der Deutschen Gesellschaft fur Pathologie 55:506-510.

Meier-Ruge WA, Bruder E, Kapur RP (2006) Intestinal neuronal dysplasia type B: one giant ganglion is not good enough. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society 9:444-452.

Meier-Ruge WA, Ammann K, Bruder E, Holschneider AM, Scharli AF, Schmittenbecher PP, Stoss F (2004) Updated results on intestinal neuronal dysplasia (IND B). European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery [et al] = Zeitschrift fur Kinderchirurgie 14:384-391.

Meir M, Burkard N, Ungewiß H, Diefenbacher M, Flemming S, Kannapin F, Germer C-T, Schweinlin M, Metzger M, Waschke J, Schlegel N (2019) Neurotrophic factor GDNF regulates intestinal barrier function in inflammatory bowel disease. The Journal of Clinical Investigation 129:2824-2840.

Memic F, Knoflach V, Morarach K, Sadler R, Laranjeira C, Hjerling-Leffler J, Sundström E, Pachnis V, Marklund U (2018) Transcription and Signaling Regulators in Developing Neuronal Subtypes of Mouse and Human Enteric Nervous System. Gastroenterology 154:624-636.

Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of Cell Fate Decision of Undifferentiated Spermatogonia by GDNF. 287:1489-1493.

Meyers EN, Lewandoski M, Martin GR (1998) An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nature genetics 18:136-141.

Mijatovic J, Piltonen M, Alberton P, Männistö PT, Saarma M, Piepponen TP (2011) Constitutive Ret signaling is protective for dopaminergic cell bodies but not for axonal terminals. Neurobiology of Aging 32:1486-1494.

Mijatovic J, Airavaara M, Planken A, Auvinen P, Raasmaja A, Piepponen TP, Costantini F, Ahtee L, Saarma M (2007) Constitutive Ret activity in knock-in multiple endocrine neoplasia type B mice induces profound elevation of brain dopamine concentration via enhanced synthesis and increases the number of TH-positive cells in the substantia nigra. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:4799-4809.

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME, Mayford M (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron 36:507-519.

Miura P, Shenker S, Andreu-Agullo C, Westholm JO, Lai EC (2013) Widespread and extensive lengthening of 3' UTRs in the mammalian brain. Genome Res 23:812-825.

Miyamoto R, Jijiwa M, Asai M, Kawai K, Ishida-Takagishi M, Mii S, Asai N, Enomoto A, Murakumo Y, Yoshimura A, Takahashi M (2011) Loss of Sprouty2 partially rescues renal hypoplasia and stomach hypoganglionosis but not intestinal aganglionosis in Ret Y1062F mutant mice. Developmental biology 349:160-168.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382:76-79.

Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB, Bousvaros A, Korzenik J, Sands BE, Xavier RJ, Huttenhower C (2012) Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome biology 13:R79-R79.

Morrison PF, Lonser RR, Oldfield EH (2007) Convective delivery of glial cell line-derived neurotrophic factor in the human putamen. Journal of neurosurgery 107:74-83.

Mulligan LM, Eng C, Attie T, Lyonnet S, Marsh DJ, Hyland VJ, Robinson BG, Frilling A, Verellen-Dumoulin C, Safar A, et al. (1994) Diverse phenotypes associated with exon 10 mutations of the RET proto-oncogene. Human molecular genetics 3:2163-2167.

Murphy F, Puri P (2005) New insights into the pathogenesis of Hirschsprung's associated enterocolitis. Pediatric surgery international 21:773-779.

Mwizerwa O, Das P, Nagy N, Akbareian SE, Mably JD, Goldstein AM (2011) Gdnf is mitogenic, neurotrophic, and chemoattractive to enteric neural crest cells in the embryonic colon. Developmental dynamics : an official publication of the American Association of Anatomists 240:1402-1411.

Mätlik K, Olfat S, Garton DR, Montaño-Rodriguez A, Turconi G, Porokuokka LL, Panhelainen A, Schweizer N, Kopra J, Cowlishaw MC, Piepponen TP, Zhang F-P, Sipilä P, Jakobsson J, Andressoo J-O (2019, manuscript) Gene Knock Up via 3'UTR editing to study gene function in vivo.775031.

Nagy A, Moens C, Ivanyi E, Pawling J, Gertsenstein M, Hadjantonakis AK, Pirity M, Rossant J (1998) Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. Current biology : CB 8:661-664.

Nagy N, Goldstein AM (2006) Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. Developmental biology 293:203-217.

Nagy N, Goldstein AM (2017) Enteric nervous system development: A crest cell's journey from neural tube to colon. Seminars in Cell & Developmental Biology 66:94-106.

Nakamura H, Lim T, Puri P (2018a) Inflammatory bowel disease in patients with Hirschsprung's disease: a systematic review and meta-analysis. Pediatric surgery international 34:149-154.

Nakamura H, Tomuschat C, Coyle D, O'Donnel AM, Lim T, Puri P (2018b) Altered goblet cell function in Hirschsprung's disease. Pediatric surgery international 34:121-128.

Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E (2002) Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129:5151-5160.

Nathanson NM (2012) Regulation of neurokine receptor signaling and trafficking. Neurochemistry international 61:874-878.

Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biology of reproduction 74:314-321.

Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW (1998) Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science (New York, NY) 279:1725-1729.

Nielsen J, Gotfryd K, Li S, Kulahin N, Soroka V, Rasmussen KK, Bock E, Berezin V (2009) Role of Glial Cell Line-Derived Neurotrophic Factor (GDNF)–Neural Cell Adhesion Molecule (NCAM) Interactions in Induction of Neurite Outgrowth and Identification of a Binding Site for NCAM in the Heel Region of GDNF. 29:11360-11376.

Nijssen J, Aguila J, Hoogstraaten R, Kee N, Hedlund E (2018) Axon-Seq Decodes the Motor Axon Transcriptome and Its Modulation in Response to ALS. Stem cell reports 11:1565-1578.

Nishiyama C, Uesaka T, Manabe T, Yonekura Y, Nagasawa T, Newgreen DF, Young HM, Enomoto H (2012) Trans-mesenteric neural crest cells are the principal source of the colonic enteric nervous system. Nature neuroscience 15:1211-1218.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws ER, Jr., Lozano AM, Penn RD, Simpson RK, Jr., Stacy M, Wooten GF (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60:69-73.

O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol (Lausanne) 9:402-402.

Obermayr F, Seitz G (2018) Recent developments in cell-based ENS regeneration - a short review. Innov Surg Sci 3:93-99.

Obermayr F, Stamp LA, Anderson CR, Young HM (2013) Genetic fate-mapping of tyrosine hydroxylase-expressing cells in the enteric nervous system. 25:e283-e291.

Oh-hashi K, Hirata Y, Kiuchi K (2012) Characterization of 3'-untranslated region of the mouse GDNF gene. BMC Mol Biol 13:2-2.

Okamoto M, Yoshioka Y, Maeda K, Bito Y, Fukumoto T, Uesaka T, Enomoto H (2019) Mice conditionally expressing RET(C618F) mutation display C cell hyperplasia and hyperganglionosis of the enteric nervous system. 57:e23292.

Oliveto S, Mancino M, Manfrini N, Biffo S (2017) Role of microRNAs in translation regulation and cancer. World J Biol Chem 8:45-56.

Ortega-de San Luis C, Pascual A (2016) Simultaneous Detection of Both GDNF and GFRα1 Expression Patterns in the Mouse Central Nervous System. 10.

Otsuka H, Fukao A, Funakami Y, Duncan KE, Fujiwara T (2019) Emerging Evidence of Translational Control by AU-Rich Element-Binding Proteins. Front Genet 10:332-332.

Pan-Montojo F, Anichtchik O, Dening Y, Knels L, Pursche S, Jung R, Jackson S, Gille G, Spillantini MG, Reichmann H, Funk RH (2010) Progression of Parkinson's disease pathology is reproduced by intragastric administration of rotenone in mice. PloS one 5:e8762.

Pan ZW, Luo CF, Liu ZJ, Li JC (2012) RET 3'UTR polymorphisms and its protective role in Hirschsprung disease in southeastern Chinese. Journal of pediatric surgery 47:1699-1705.

Paratcha G, Ledda F (2008) GDNF and GFRalpha: a versatile molecular complex for developing neurons. Trends in neurosciences 31:384-391.

Paratcha G, Ledda F, Ibanez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113:867-879.

Parisi MA, Baldessari AE, Iida MHK, Clarke CM, Doggett B, Shirasawa S, Kapur RP (2003) Genetic background modifies intestinal pseudo-obstruction and the expression of a reporter gene in Hox11L1−/− mice1 1The authors thank Carmen Booth for technical assistance and Kristy Seidel for statistical analysis. Gastroenterology 125:1428-1440.

Pascual A, Hidalgo-Figueroa M, Piruat JI, Pintado CO, Gomez-Diaz R, Lopez-Barneo J (2008) Absolute requirement of GDNF for adult catecholaminergic neuron survival. Nature neuroscience 11:755-761.

Patel BA, Patel N, Fidalgo S, Wang C, Ranson RN, Saffrey MJ, Yeoman MS (2014) Impaired colonic motility and reduction in tachykinin signalling in the aged mouse. Experimental Gerontology 53:24-30.

Pedrosa Carrasco AJ, Timmermann L, Pedrosa DJ (2018) Management of constipation in patients with Parkinson's disease. NPJ Parkinsons Dis 4:6-6.

Pei L-y, Ke Y-s, Zhao H-h, Wang L, Jia C, Liu W-z, Fu Q-h, Shi M-n, Cui J, Li S-c (2019) Role of colonic microbiota in the pathogenesis of ulcerative colitis. BMC Gastroenterol 19:10.

Pelaseyed T, Bergstrom JH, Gustafsson JK, Ermund A, Birchenough GM, Schutte A, van der Post S, Svensson F, Rodriguez-Pineiro AM, Nystrom EE, Wising C, Johansson ME, Hansson GC (2014) The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunological reviews 260:8-20.

Peng X, Varendi K, Maimets M, Andressoo JO, Coppes RP (2017) Role of glial-cell-derived neurotrophic factor in salivary gland stem cell response to irradiation. Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology 124:448-454.

Perea D, Guiu J, Hudry B, Konstantinidou C, Milona A, Hadjieconomou D, Carroll T, Hoyer N, Natarajan D, Kallijärvi J, Walker JA, Soba P, Thapar N, Burns AJ, Jensen KB, Miguel-Aliaga I (2017) Ret receptor tyrosine kinase sustains proliferation and tissue maturation in intestinal epithelia. The EMBO journal 36:3029-3045.

Peters RJ, Osinski MA, Hongo JA, Bennett GL, Okragly AJ, Haak-Frendscho M, Epstein ML (1998) GDNF is abundant in the adult rat gut. Journal of the autonomic nervous system 70:115-122.

Pham TD, Gershon MD, Rothman TP (1991) Time of origin of neurons in the murine enteric nervous system: Sequence in relation to phenotype. 314:789-798.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382:73-76.

Piletič K, Kunej TJAoT (2016) MicroRNA epigenetic signatures in human disease. 90:2405-2419.

Plaza-Menacho I, Burzynski GM, de Groot JW, Eggen BJ, Hofstra RM (2006) Current concepts in RET-related genetics, signaling and therapeutics. Trends in genetics : TIG 22:627-636.

Plaza-Menacho I, van der Sluis T, Hollema H, Gimm O, Buys CH, Magee AI, Isacke CM, Hofstra RM, Eggen BJ (2007) Ras/ERK1/2-mediated STAT3 Ser727 phosphorylation by familial medullary thyroid carcinoma-associated RET mutants induces full activation of STAT3 and is required for c-fos promoter activation, cell mitogenicity, and transformation. The Journal of biological chemistry 282:6415-6424.

Poirier A-A, Aubé B, Côté M, Morin N, Di Paolo T, Soulet D (2016) Gastrointestinal Dysfunctions in Parkinson's Disease: Symptoms and Treatments. Parkinsons Dis 2016:6762528-6762528.

Pomeranz HD, Gershon MD (1990) Colonization of the avian hindgut by cells derived from the sacral neural crest. Developmental biology 137:378-394.

Popsueva A, Poteryaev D, Arighi E, Meng X, Angers-Loustau A, Kaplan D, Saarma M, Sariola H (2003) GDNF promotes tubulogenesis of GFRalpha1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. The Journal of cell biology 161:119-129.

Prelich G (2012) Gene overexpression: uses, mechanisms, and interpretation. Genetics 190:841-854.

Puffenberger EG, Kauffman ER, Bolk S, Matise TC, Washington SS, Angrist M, Weissenbach J, Garver KL, Mascari M, Ladda R, et al. (1994) Identity-by-descent and association mapping of a recessive gene for Hirschsprung disease on human chromosome 13q22. Human molecular genetics 3:1217-1225.

Puig I, Champeval D, De Santa Barbara P, Jaubert F, Lyonnet S, Larue L (2009) Deletion of Pten in the mouse enteric nervous system induces ganglioneuromatosis and mimics intestinal pseudoobstruction. The Journal of clinical investigation 119:3586-3596.

Puri P, Rolle U (2004) Variant Hirschsprung's disease. Seminars in pediatric surgery 13:293-299.

Ramalho-Santos M, Melton DA, McMahon AP (2000) Hedgehog signals regulate multiple aspects of gastrointestinal development. 127:2763-2772.

Rao M, Gershon MD (2016) The bowel and beyond: the enteric nervous system in neurological disorders. Nature reviews Gastroenterology & hepatology 13:517-528.

Raveenthiran V (2011) Knowledge of ancient Hindu surgeons on Hirschsprung disease: evidence from Sushruta Samhita of circa 1200-600 BC. Journal of pediatric surgery 46:2204-2208.

Ray P, Tang W, Wang P, Homer R, Kuhn C, 3rd, Flavell RA, Elias JA (1997) Regulated overexpression of interleukin 11 in the lung. Use to dissociate development-dependent and -independent phenotypes. J Clin Invest 100:2501-2511.

Rietdijk CD, Perez-Pardo P, Garssen J, van Wezel RJA, Kraneveld AD (2017) Exploring Braak's Hypothesis of Parkinson's Disease. Front Neurol 8:37-37.

Robertson BR, O'Rourke JL, Neilan BA, Vandamme P, On SL, Fox JG, Lee A (2005) Mucispirillum schaedleri gen. nov., sp. nov., a spiral-shaped bacterium colonizing the mucus layer of the gastrointestinal tract of laboratory rodents. International journal of systematic and evolutionary microbiology 55:1199-1204.

Rodrigues DM, Li AY, Nair DG, Blennerhassett MG (2011) Glial cell line-derived neurotrophic factor is a key neurotrophin in the postnatal enteric nervous system. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 23:e44-56.

Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, Ceccherini I, Pasini B, Bocciardi R, Lerone M, Kääriäinen H, Martucciello G (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 367:377-378.

Rooks MG, Veiga P, Wardwell-Scott LH, Tickle T, Segata N, Michaud M, Gallini CA, Beal C, van Hylckama-Vlieg JE, Ballal SA, Morgan XC, Glickman JN, Gevers D, Huttenhower C, Garrett WS (2014) Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. Isme j 8:1403-1417.

Rose C, Parker A, Jefferson B, Cartmell E (2015) The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. Crit Rev Environ Sci Technol 45:1827-1879.

Rota L, Pellegrini C, Benvenuti L, Antonioli L, Fornai M, Blandizzi C, Cattaneo A, Colla E (2019) Constipation, deficit in colon contractions and alpha-synuclein inclusions within the colon precede motor abnormalities and neurodegeneration in the central nervous system in a mouse model of alpha-synucleinopathy. Transl Neurodegener 8:5-5.

Rotem N, Magen I, Ionescu A, Gershoni-Emek N, Altman T, Costa CJ, Gradus T, Pasmanik-Chor M, Willis DE, Ben-Dov IZ, Hornstein E, Perlson E (2017) ALS Along the Axons - Expression of Coding and Noncoding RNA Differs in Axons of ALS models. Scientific reports 7:44500.

Rothman TP, Gershon MD (1982) Phenotypic expression in the developing murine enteric nervous system. The Journal of neuroscience : the official journal of the Society for Neuroscience 2:381-393.

Ruiz-Ferrer M, Torroglosa A, Luzon-Toro B, Fernandez RM, Antinolo G, Mulligan LM, Borrego S (2011) Novel mutations at RET ligand genes preventing receptor activation are associated to Hirschsprung's disease. Journal of molecular medicine (Berlin, Germany) 89:471-480.

Ruther U, Garber C, Komitowski D, Muller R, Wagner EF (1987) Deregulated c-fos expression interferes with normal bone development in transgenic mice. Nature 325:412-416.

Ryan ET, Ecker JL, Christakis NA, Folkman J (1992) Hirschsprung's disease: associated abnormalities and demography. Journal of pediatric surgery 27:76-81.

Saarenpaa T, Kogan K, Sidorova Y, Mahato AK, Tascon I, Kaljunen H, Yu L, Kallijarvi J, Jurvansuu J, Saarma M, Goldman A (2017) Zebrafish GDNF and its co-receptor GFRalpha1 activate the human RET receptor and promote the survival of dopaminergic neurons in vitro. PloS one 12:e0176166.

Sadowski PD (1995) The Flp recombinase of the 2-microns plasmid of Saccharomyces cerevisiae. Progress in nucleic acid research and molecular biology 51:53-91.

Saffrey MJ (2013) Cellular changes in the enteric nervous system during ageing. Developmental biology 382:344-355.

Sainio K, Suvanto P, Davies J, Wartiovaara J, Wartiovaara K, Saarma M, Arumae U, Meng X, Lindahl M, Pachnis V, Sariola H (1997) Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. 124:4077-4087.

Sakakibara R, Odaka T, Uchiyama T, Asahina M, Yamaguchi K, Yamaguchi T, Yamanishi T, Hattori T (2003) Colonic transit time and rectoanal videomanometry in Parkinson's disease. Journal of neurology, neurosurgery, and psychiatry 74:268-272.

Salmena L, Carracedo A, Pandolfi PP (2008) Tenets of PTEN Tumor Suppression. Cell 133:403-414.

Salomon R, Attie T, Pelet A, Bidaud C, Eng C, Amiel J, Sarnacki S, Goulet O, Ricour C, Nihoul-Fekete C, Munnich A, Lyonnet S (1996) Germline mutations of the RET ligand GDNF are not sufficient to cause Hirschsprung disease. Nature genetics 14:345-347.

Salvatore MF, Ai Y, Fischer B, Zhang AM, Grondin RC, Zhang Z, Gerhardt GA, Gash DM (2006) Point source concentration of GDNF may explain failure of phase II clinical trial. Experimental neurology 202:497-505.

Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382:70-73.

Santos R, Ursu O, Gaulton A, Bento AP, Donadi RS, Bologa CG, Karlsson A, Al-Lazikani B, Hersey A, Oprea TI, Overington JP (2016) A comprehensive map of molecular drug targets. Nature Reviews Drug Discovery 16:19.

Saragovi HU, Galan A, Levin LA (2019) Neuroprotection: Pro-survival and Anti-neurotoxic Mechanisms as Therapeutic Strategies in Neurodegeneration. Frontiers in cellular neuroscience 13:231-231.

Sasselli V, Pachnis V, Burns AJ (2012) The enteric nervous system. Developmental biology 366:64-73.

Savica R, Carlin JM, Grossardt BR, Bower JH, Ahlskog JE, Maraganore DM, Bharucha AE, Rocca WA (2009) Medical records documentation of constipation preceding Parkinson disease: A case-control study. Neurology 73:1752-1758.

Schenck Eidam H et al. (2018) Discovery of a First-in-Class Gut-Restricted RET Kinase Inhibitor as a Clinical Candidate for the Treatment of IBS. ACS medicinal chemistry letters 9:623-628.

Schimpl G, Uray E, Ratschek M, Hollwarth ME (2004) Constipation and intestinal neuronal dysplasia type B: a clinical follow-up study. Journal of pediatric gastroenterology and nutrition 38:308-311.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367:380-383.

Schwerk J, Savan R (2015) Translating the Untranslated Region. 195:2963-2971.

Seelig DM, Mason GL, Telling GC, Hoover EA (2011) Chronic wasting disease prion trafficking via the autonomic nervous system. Am J Pathol 179:1319-1328.

Sharkey KA (2015) Emerging roles for enteric glia in gastrointestinal disorders. The Journal of clinical investigation 125:918-925.

Sharma S, Lu H-C (2018) microRNAs in Neurodegeneration: Current Findings and Potential Impacts. J Alzheimers Dis Parkinsonism 8:420.

Shen L, Pichel JG, Mayeli T, Sariola H, Lu B, Westphal H (2002) Gdnf haploinsufficiency causes Hirschsprung-like intestinal obstruction and early-onset lethality in mice. American journal of human genetics 70:435-447.

Shepherd IT, Beattie CE, Raible DW (2001) Functional analysis of zebrafish GDNF. Developmental biology 231:420-435.

Shi C, Welsh PA, Sass-Kuhn S, Wang X, McCloskey DE, Pegg AE, Feith DJ (2012) Characterization of transgenic mice with overexpression of spermidine synthase. Amino Acids 42:495-505.

Sidorova YA, Matlik K, Paveliev M, Lindahl M, Piranen E, Milbrandt J, Arumae U, Saarma M, Bespalov MM (2010) Persephin signaling through GFRalpha1: the potential for the treatment of Parkinson's disease. Molecular and cellular neurosciences 44:223-232.

Sidorova YA, Bespalov MM, Wong AW, Kambur O, Jokinen V, Lilius TO, Suleymanova I, Karelson G, Rauhala PV, Karelson M, Osborne PB, Keast JR, Kalso EA, Saarma M (2017) A Novel Small Molecule GDNF Receptor RET Agonist, BT13, Promotes Neurite Growth from Sensory Neurons in Vitro and Attenuates Experimental Neuropathy in the Rat. Front Pharmacol 8:365-365.

Siepel A, Bejerano G, Pedersen JS, Hinrichs AS, Hou M, Rosenbloom K, Clawson H, Spieth J, Hillier LW, Richards S, Weinstock GM, Wilson RK, Gibbs RA, Kent WJ, Miller W, Haussler D (2005) Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res 15:1034-1050.

Simola N, Morelli M, Carta AR (2007) The 6-hydroxydopamine model of Parkinson's disease. Neurotoxicity research 11:151-167.

Slevin JT, Gash DM, Smith CD, Gerhardt GA, Kryscio R, Chebrolu H, Walton A, Wagner R, Young AB (2007) Unilateral intraputamenal glial cell line-derived neurotrophic factor in patients with Parkinson disease: response to 1 year of treatment and 1 year of withdrawal. Journal of neurosurgery 106:614-620.

Smith-Hicks CL, Sizer KC, Powers JF, Tischler AS, Costantini F (2000) C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B. The EMBO journal 19:612-622.

Smith JF, Mahmood S, Song F, Morrow A, Smiraglia D, Zhang X, Rajput A, Higgins MJ, Krumm A, Petrelli NJ, Costello JF, Nagase H, Plass C, Held W (2007) Identification of DNA Methylation in 3' Genomic Regions that are Associated with Upregulation of Gene Expression in Colorectal Cancer. Epigenetics 2:161-172.

Smith ZD, Meissner A (2013) DNA methylation: roles in mammalian development. Nature reviews Genetics 14:204-220.

Southard-Smith EM, Kos L, Pavan WJ (1998) Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nature genetics 18:60-64.

Spitz F, Duboule D (2008) Global control regions and regulatory landscapes in vertebrate development and evolution. Advances in genetics 61:175-205.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW (1998) Negative Regulation of PKB/Akt-Dependent Cell Survival by the Tumor Suppressor PTEN. Cell 95:29-39.

Steinkamp M, Gundel H, Schulte N, Spaniol U, Pflueger C, Zizer E, von Boyen GBT (2012) GDNF protects enteric glia from apoptosis: evidence for an autocrine loop. BMC Gastroenterol 12:6-6.

Stojanovska V, McQuade RM, Miller S, Nurgali K (2018) Effects of Oxaliplatin Treatment on the Myenteric Plexus Innervation and Glia in the Murine Distal Colon. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 66:723-736.

Sumida K, Molnar MZ, Potukuchi PK, Thomas F, Lu JL, Matsushita K, Yamagata K, Kalantar-Zadeh K, Kovesdy CP (2017) Constipation and Incident CKD. J Am Soc Nephrol 28:1248-1258.

Sun S, Lei Y, Li Q, Wu Y, Zhang L, Mu P-P, Ji G-Q, Tang C-X, Wang Y-Q, Gao J, Gao J, Li L, Zhuo L, Li Y-Q, Gao D-S (2017) Neuropilin-1 is a glial cell line-derived neurotrophic factor receptor in glioblastoma. Oncotarget 8:74019-74035.

Tahira T, Ishizaka Y, Itoh F, Sugimura T, Nagao M (1990) Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene 5:97-102.

Taketomi T, Yoshiga D, Taniguchi K, Kobayashi T, Nonami A, Kato R, Sasaki M, Sasaki A, Ishibashi H, Moriyama M, Nakamura K-i, Nishimura J, Yoshimura A (2005) Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and esophageal achalasia. Nature neuroscience 8:855-857.

Tam PK, Boyd GP (1990) Origin, course, and endings of abnormal enteric nerve fibres in Hirschsprung's disease defined by whole-mount immunohistochemistry. Journal of pediatric surgery 25:457-461.

Tanner CM, Ross GW, Jewell SA, Hauser RA, Jankovic J, Factor SA, Bressman S, Deligtisch A, Marras C, Lyons KE, Bhudhikanok GS, Roucoux DF, Meng C, Abbott RD, Langston JW (2009) Occupation and risk of parkinsonism: a multicenter case-control study. Archives of neurology 66:1106-1113.

Tate PH, Bird AP (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. Current opinion in genetics & development 3:226-231.

Tautz D (1992) Problems and paradigms: Redundancies, development and the flow of information. 14:263-266.

Teitelbaum DH, Caniano DA, Qualman SJ (1989) The pathophysiology of Hirschsprung's-associated enterocolitis: importance of histologic correlates. Journal of pediatric surgery 24:1271-1277.

Thiagarajah JR, Yildiz H, Carlson T, Thomas AR, Steiger C, Pieretti A, Zukerberg LR, Carrier RL, Goldstein AM (2014) Altered goblet cell differentiation and surface mucus properties in Hirschsprung disease. PloS one 9:e99944-e99944.

Tian B, Manley JL (2017) Alternative polyadenylation of mRNA precursors. Nat Rev Mol Cell Biol 18:18-30.

Toledo de Arruda Lourenção PL, Terra SA, Ortolan EVP, Rodrigues MAM (2016) Intestinal neuronal dysplasia type B: A still little known diagnosis for organic causes of intestinal chronic constipation. World journal of gastrointestinal pharmacology and therapeutics 7:397-405.

Tomuschat C, Puri PJPSI (2015) RET gene is a major risk factor for Hirschsprung's disease: a meta-analysis. 31:701-710.

Torroglosa A, Villalba-Benito L, Luzón-Toro B, Fernández RM, Antiñolo G, Borrego S (2019) Epigenetic Mechanisms in Hirschsprung Disease. Int J Mol Sci 20:3123.

Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. Nature 382:80-83.

Trendelenburg P (2006) Physiological and pharmacological investigations of small intestinal peristalsis. Translation of the article "Physiologische und pharmakologische Versuche uber die Dunndarmperistaltik", Arch. Exp. Pathol. Pharmakol. 81, 55-129, 1917. Naunyn-Schmiedeberg's archives of pharmacology 373:101-133.

Trupp M, Belluardo N, Funakoshi H, Ibanez CF (1997) Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:3554-3567.

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, Ibanez CF (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. The Journal of cell biology 130:137-148.

Turconi G, Kopra J, Võikar V, Kulesskaya N, Vilenius C, Piepponen TP, Andressoo J-O (2020) Chronic two-fold elevation of endogenous GDNF levels is safe and enhances motor and dopaminergic function in aged mice. Molecular Therapy - Methods & Clinical Development.

Uesaka T, Nagashimada M, Enomoto H (2013) GDNF signaling levels control migration and neuronal differentiation of enteric ganglion precursors. The Journal of neuroscience : the official journal of the Society for Neuroscience 33:16372-16382.

Uesaka T, Nagashimada M, Enomoto H (2015) Neuronal Differentiation in Schwann Cell Lineage Underlies Postnatal Neurogenesis in the Enteric Nervous System. 35:9879-9888.

Uesaka T, Nagashimada M, Yonemura S, Enomoto H (2008) Diminished Ret expression compromises neuronal survival in the colon and causes intestinal aganglionosis in mice. The Journal of clinical investigation 118:1890-1898.

Uesaka T, Jain S, Yonemura S, Uchiyama Y, Milbrandt J, Enomoto H (2007) Conditional ablation of GFRalpha1 in postmigratory enteric neurons triggers unconventional neuronal death in the colon and causes a Hirschsprung's disease phenotype. Development 134:2171-2181.

Ure BM, Holschneider AM, Meier-Ruge W (1994) Neuronal Intestinal Malformations: A Retro- and Prospective Study on 203 Patients. European journal of pediatric surgery : official journal of Austrian Association of Pediatric Surgery [et al] = Zeitschrift fur Kinderchirurgie 4:279-286.

Wallace AS, Barlow AJ, Navaratne L, Delalande J-m, Tauszig-delamasure S, Corset V, Thapar N, Burns AJ (2009) Inhibition of cell death results in hyperganglionosis: implications for enteric nervous system development. 21:768-e749.

Van Den Berge N, Ferreira N, Gram H, Mikkelsen TW, Alstrup AKO, Casadei N, Tsung-Pin P, Riess O, Nyengaard JR, Tamguney G, Jensen PH, Borghammer P (2019) Evidence for bidirectional and trans-synaptic parasympathetic and sympathetic propagation of alpha-synuclein in rats. Acta neuropathologica 138:535-550.

Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J (2016) Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates. 65:57-62.

Vanderwinden JM, De Laet MH, Schiffmann SN, Mailleux P, Lowenstein CJ, Snyder SH, Vanderhaeghen JJ (1993) Nitric oxide synthase distribution in the enteric nervous system of Hirschsprung's disease. Gastroenterology 105:969-973.

Wang B, He L, Dong H, Dalton TP, Nebert DW (2011) Generation of a Slc39a8 hypomorph mouse: markedly decreased ZIP8 Zn^{2+}/$(HCO_3^-)_2$ transporter expression. Biochemical and biophysical research communications 410:289-294.

Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, Wu X, Vo HT, Ma XJ, Luo Y (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. The Journal of molecular diagnostics : JMD 14:22-29.

Wang H, Hughes I, Planer W, Parsadanian A, Grider JR, Vohra BP, Keller-Peck C, Heuckeroth RO (2010) The timing and location of glial cell line-derived neurotrophic factor expression determine enteric nervous system structure and function. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:1523-1538.

Wang IK, Lin CL, Wu YY, Chou CY, Lin SY, Liu JH, Yen TH, Huang CC, Sung FC (2014) Increased risk of Parkinson's disease in patients with end-stage renal disease: a retrospective cohort study. Neuroepidemiology 42:204-210.

Wang L, Tanaka Y, Wang D, Morikawa M, Zhou R, Homma N, Miyamoto Y, Hirokawa N (2018) The Atypical Kinesin KIF26A Facilitates Termination of Nociceptive Responses by Sequestering Focal Adhesion Kinase. Cell reports 24:2894-2907.

Waterston RH et al. (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420:520-562.

Webster W (1973) Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. Journal of embryology and experimental morphology 30:573-585.

Webster W (1974) Aganglionic megacolon in piebald-lethal mice. Archives of pathology 97:111-117.

Wedel T, Busing V, Heinrichs G, Nohroudi K, Bruch HP, Roblick UJ, Bottner M (2010) Diverticular disease is associated with an enteric neuropathy as revealed by morphometric analysis. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 22:407-414, e493-404.

Wei Y, Zhou X, Fang C, Li L, Kluetzman K, Yang W, Zhang QY, Ding X (2010) Generation of a mouse model with a reversible hypomorphic cytochrome P450 reductase gene: utility for tissue-specific rescue of the reductase expression, and insights from a resultant mouse model with global suppression of P450 reductase expression in extrahepatic tissues. The Journal of pharmacology and experimental therapeutics 334:69-77.

Wen J, Parker BJ, Jacobsen A, Krogh A (2011) MicroRNA transfection and AGO-bound CLIP-seq data sets reveal distinct determinants of miRNA action. RNA 17:820-834.

Verity AN, Wyatt TL, Hajos B, Eglen RM, Baecker PA, Johnson RM (1998) Regulation of Glial Cell Line-Derived Neurotrophic Factor Release from Rat C6 Glioblastoma Cells. 70:531-539.

Verity AN, Wyatt TL, Lee W, Hajos B, Baecker PA, Eglen RM, Johnson RM (1999) Differential regulation of glial cell line–derived neurotrophic factor (GDNF) expression in human neuroblastoma and glioblastoma cell lines. 55:187-197.

Whone A et al. (2019a) Randomized trial of intermittent intraputamenal glial cell line-derived neurotrophic factor in Parkinson's disease. Brain : a journal of neurology 142:512-525.

Whone AL et al. (2019b) Extended Treatment with Glial Cell Line-Derived Neurotrophic Factor in Parkinson's Disease. Journal of Parkinson's disease 9:301-313.

Wiesenhofer B, Stockhammer G, Kostron H, Maier H, Hinterhuber H, Humpel CJAN (2000) Glial cell line-derived neurotrophic factor (GDNF) and its receptor (GFR-α1) are strongly expressed in human gliomas. 99:131-137.

Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75:855-862.

Vital M, Howe AC, Tiedje JM (2014) Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. 5:e00889-00814.

Wittkopp PJ, Kalay G (2011) Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nature reviews Genetics 13:59-69.

Vogler C, Galvin N, Levy B, Grubb J, Jiang J, Zhou XY, Sly WS (2003) Transgene produces massive overexpression of human beta -glucuronidase in mice, lysosomal storage of enzyme, and strain-dependent tumors. Proceedings of the National Academy of Sciences of the United States of America 100:2669-2673.

Wojciechowska A, Braniewska A, Kozar-Kaminska K (2017) MicroRNA in cardiovascular biology and disease. Advances in clinical and experimental medicine : official organ Wroclaw Medical University 26:865-874.

Wolpowitz D, Mason TB, Dietrich P, Mendelsohn M, Talmage DA, Role LW (2000) Cysteine-rich domain isoforms of the neuregulin-1 gene are required for maintenance of peripheral synapses. Neuron 25:79-91.

von Boyen GB, Krammer HJ, Suss A, Dembowski C, Ehrenreich H, Wedel T (2002) Abnormalities of the enteric nervous system in heterozygous endothelin B receptor deficient (spotting lethal) rats resembling intestinal neuronal dysplasia. Gut 51:414-419.

von Boyen GBT, Schulte N, Pflüger C, Spaniol U, Hartmann C, Steinkamp M (2011) Distribution of enteric glia and GDNF during gut inflammation. BMC Gastroenterol 11:3-3.

Worley DS, Pisano JM, Choi ED, Walus L, Hession CA, Cate RL, Sanicola M, Birren SJ (2000) Developmental regulation of GDNF response and receptor expression in the enteric nervous system. Development 127:4383-4393.

Wu L, Gu J, Cui H, Zhang QY, Behr M, Fang C, Weng Y, Kluetzman K, Swiatek PJ, Yang W, Kaminsky L, Ding X (2005) Transgenic mice with a hypomorphic NADPH-cytochrome P450 reductase gene: effects on development, reproduction, and microsomal cytochrome P450. The Journal of pharmacology and experimental therapeutics 312:35-43.

Wu S, Zhang YG, Lu R, Xia Y, Zhou D, Petrof EO, Claud EC, Chen D, Chang EB, Carmeliet G, Sun J (2015) Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. Gut 64:1082-1094.

Xiao N, Lin Y, Cao H, Sirjani D, Giaccia AJ, Koong AC, Kong CS, Diehn M, Le QT (2014) Neurotrophic factor GDNF promotes survival of salivary stem cells. J Clin Invest 124:3364-3377.

Xie X, Lu J, Kulbokas EJ, Golub TR, Mootha V, Lindblad-Toh K, Lander ES, Kellis M (2005) Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. Nature 434:338-345.

Yamada T, Ohtani S, Sakurai T, Tsuji T, Kunieda T, Yanagisawa M (2006) Reduced Expression of the Endothelin Receptor Type B Gene in Piebald Mice Caused by Insertion of a Retroposon-like Element in Intron 1. 281:10799-10807.

Yamataka A, Hatano M, Kobayashi H, Wang K, Miyahara K, Sueyoshi N, Miyano T (2001) Intestinal neuronal dysplasia-like pathology in Ncx/Hox11L.1 gene-deficient mice. Journal of pediatric surgery 36:1293-1296.

Yanagisawa H, Yanagisawa M, Kapur RP, Richardson JA, Williams SC, Clouthier DE, de Wit D, Emoto N, Hammer RE (1998) Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. Development 125:825-836.

Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J, Swinton PG, Martin JF, Behringer RR (2014) Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Developmental biology 393:3-9.

Young HM, Jones BR, McKeown SJ (2002) The projections of early enteric neurons are influenced by the direction of neural crest cell migration. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:6005-6018.

Young HM, Hearn CJ, Ciampoli D, Southwell BR, Brunet JF, Newgreen DF (1998) A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture. Developmental biology 202:67-84.

Young HM, Hearn CJ, Farlie PG, Canty AJ, Thomas PQ, Newgreen DF (2001) GDNF is a chemoattractant for enteric neural cells. Developmental biology 229:503-516.

Yu T, Scully S, Yu Y, Fox GM, Jing S, Zhou R (1998) Expression of GDNF Family Receptor Components during Development: Implications in the Mechanisms of Interaction. 18:4684-4696.

Zbuk KM, Eng C (2006) Cancer phenomics: RET and PTEN as illustrative models. Nature Reviews Cancer 7:35.

Zeisel A, Hochgerner H, Lönnerberg P, Johnsson A, Memic F, van der Zwan J, Häring M, Braun E, Borm LE, La Manno G, Codeluppi S, Furlan A, Lee K, Skene N, Harris KD, Hjerling-Leffler J, Arenas E, Ernfors P, Marklund U, Linnarsson S (2018) Molecular Architecture of the Mouse Nervous System. Cell 174:999-1014.e1022.

Zhang DK, He FQ, Li TK, Pang XH, Cui DJ, Xie Q, Huang XL, Gan HT (2010) Glial-derived neurotrophic factor regulates intestinal epithelial barrier function and inflammation and is therapeutic for murine colitis. The Journal of pathology 222:213-222.

Zhang X-H, Tee LY, Wang X-G, Huang Q-S, Yang S-H (2015) Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. Molecular Therapy - Nucleic Acids 4:e264.

Zhang Y, Niswander L (2013) Zic2 is required for enteric nervous system development and neurite outgrowth: a mouse model of enteric hyperplasia and dysplasia. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 25:538-541.

Zhao Z, Alam S, Oppenheim RW, Prevette DM, Evenson A, Parsadanian A (2004) Overexpression of glial cell line-derived neurotrophic factor in the CNS rescues motoneurons from programmed cell death and promotes their long-term survival following axotomy. Experimental neurology 190:356-372.

Zhou R, Niwa S, Homma N, Takei Y, Hirokawa N (2009) KIF26A is an unconventional kinesin and regulates GDNF-Ret signaling in enteric neuronal development. Cell 139:802-813.

Zhu S, Zhao C, Wu Y, Yang Q, Shao A, Wang T, Wu J, Yin Y, Li Y, Hou J, Zhang X, Zhou G, Gu X, Wang X, Bustelo XR, Zhou J (2015) Identification of a Vav2-dependent mechanism for GDNF/Ret control of mesolimbic DAT trafficking. Nature neuroscience 18:1084-1093.

ISBN 978-951-51-6074-4 (PRINT)
ISBN 978-951-51-6075-1 (ONLINE)
ISSN 2342-3161 (PRINT)
ISSN 2342-317X (ONLINE)
http://ethesis.helsinki.fi

HELSINKI 2020